OXYGEN FREE RADICALS AND MODULATION OF ILEUM SMOOTH MUSCLE TONE

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University of Saskatchewan
Saskatoon

by
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SUMMARY OF DISSERTATION

Submitted in partial fulfillment
of the requirement for the
DEGREE OF DOCTOR OF PHILOSOPHY

by

Nastaran Eizadi-Mood
Toxicology Graduate Program
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Fall, 1998

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OXYGEN FREE RADICALS AND MODULATION OF ILEUM SMOOTH MUSCLE TONE

The results of this study indicate that exogenously-generated oxy-radicals produce relaxation, contraction or contraction/relaxation of rat ileum depending upon the generating system. Xanthine (X) plus xanthine oxidase (XO) produced relaxation of ileum. The relaxation was attenuated by the hydroxyl radical scavengers mannitol and dimethylthiourea (DMTU); and the singlet oxygen scavenger, histidine, supporting the idea that the hydroxyl radical ($\cdot OH$) and singlet oxygen are involved in X/XO-induced relaxation of ileum. Deferoxamine, an iron chelator, reduced the relaxation of the ileum, indicating that $\cdot OH$ mediates the X/XO-induced relaxation of ileum. Xanthine/xanthine oxidase-induced relaxation is partly mediated through cyclooxygenase metabolites, and partly through nitric oxide and ATP-sensitive potassium channels.

Hydrogen peroxide (H_2O_2) produced a biphasic response (an initial contraction followed by relaxation), which appeared to be due to the generation of $\cdot OH$. The contraction/relaxation induced by H_2O_2 is mediated through cyclooxygenase metabolites. The hydrogen peroxide-evoked biphasic response in rat ileum is not mediated through nitric oxide, acetylcholine or histamine.

Dihydroxy fumaric acid (DHF) plus ferric chloride ($FeCl_3$) and adenosine diphosphate (ADP) produced concentration-dependent contraction of ileum. Mannitol, DMTU, and histidine partially inhibited the DHF/$FeCl_3$-ADP-induced contraction, supporting the idea that hydroxyl radicals and singlet oxygen are involved in DHF/$FeCl_3$-ADP-induced contraction of ileum. DHF/$FeCl_3$-ADP-induced contraction is mediated partly through arachidonic acid metabolites and histamine. These results suggest that oxygen radicals play a role in the motility of ileum.

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ABSTRACT

The effects of oxygen-derived free radicals on rat ileum strips were investigated. Xanthine (X) plus xanthine oxidase (XO) produced relaxation of ileum. The failure of superoxide dismutase (a scavenger of superoxide anion) and catalase (a scavenger of hydrogen peroxide) to protect ileum from effects of X/XO suggest that neither superoxide anion nor hydrogen peroxide are involved in X/XO-induced relaxation of ileum. The relaxation was attenuated by the hydroxyl radical scavengers, mannitol and dimethylthiourea (DMTU) and by the singlet oxygen scavenger, histidine, supporting the idea that the hydroxyl radical and the singlet oxygen are involved in X/XO-induced relaxation of ileum. Deferoxamine, an iron chelator, reduced the relaxation of ileum, indicating that iron plays an important role in mediating the X/XO-induced relaxation of ileum. Xanthine/xanthine oxidase-induced relaxation was mainly dependent on cyclooxygenase metabolites, because indomethacin, a cyclooxygenase inhibitor, reduced the relaxation. The relaxation also appears to be partially dependent on nitric oxide and ATP-sensitive potassium channels, because N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase, and glibenclamide , an inhibitor of ATP-sensitive potassium channels, partially reduced the relaxation.

Hydrogen peroxide (H_2O_2) produced a biphasic response (an initial contraction followed by relaxation). The findings

that two hydroxyl radical scavengers, DMTU and mannitol, offered protection against the H_2O_2-induced biphasic response of ileum suggest site-specific formation of hydroxyl radical within the cell. The contraction and relaxation induced by H_2O_2 was shown to be dependent on cyclooxygenase metabolites, because indomethacin, a cyclooxygenase inhibitor, reduced the biphasic response induced by H_2O_2. In addition, the H_2O_2-induced contraction is reduced by glibenclamide, leading further support to the role of prostaglandins. The hydrogen peroxide-evoked biphasic response in rat ileum was resistant to L-NMMA (inhibitor of nitric oxide synthase), methylene blue (inhibitor of guanylate cyclase), atropine (antimuscarinic drug) and pyrilamine (histamine receptor antagonist). This suggests that nitric oxide, cGMP, acetylcholine, and histamine are not involved in mediating the H_2O_2 responses in rat ileum.

Dihydroxy fumarate (DHF) plus ferric chloride ($FeCl_3$) and adenosine diphosphate (ADP) produced concentration-dependent contraction of ileum. Mannitol , DMTU and histidine partially inhibited the DHF/$FeCl_3$-ADP-induced contraction, supporting the idea that hydroxyl radicals and singlet oxygen are involved in DHF/$FeCl_3$-ADP-induced contraction of ileum. Contraction was partially abolished by indomethacin or pyrilamine, suggesting that DHF/$FeCl_3$-ADP-induced contraction is mediated partially through arachidonic acid metabolites and histamine.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES OF APPENDIX

LIST OF ABBREVIATIONS

Symbol	Meaning
AA	arachidonic acid
ACh	acetylcholine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ca^{2+}	calcium ion
CAT	catalase
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CuZn-SOD	copper-zinc superoxide dismutase
DHF/$FeCl_3$-ADP	dihydroxy fumaric acid+ferric chloride+adenosine diphosphate
DMSO	dimethyl sulfoxide
DMTU	dimethylthiourea
DNA	deoxyribonucleic acid
ECA	electrical control activity
ECL	enterochromaffin cells
EC-SOD	extracellular superoxide dismutase
ENS	enteric nervous system
EP	prostaglandin E receptor
EP_1	prostaglandin E_1 receptor
EP_2	prostaglandin E_2 receptor
EP_3	prostaglandin E_3 receptor
Fe^{+2}	ferrous ion
Fe^{+3}	ferric ion
FP	prostaglandin F receptor
GI	gastrointestinal
GS.	thiyl radical of glutathione
Gs_{ox}	cysteic acid
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione

GTP	guanosine triphosphate
H_1	histamine receptor I
H_2	histamine receptor II
HDC	histidine decarboxylase
HO_2.	hydroperoxyl radical
H_2O	water
H_2O_2	hydrogen peroxide
HETE	hydroxy-eicosatetraeonic acid
HOCl	hypochlorous acid
HPETE	hydroperoxy-eicosatetraenoic acid
5-HT	5-hydroxy tryptamine
HX/XO	hypoxanthine/xanthine oxidase
IBD	inflammatory bowel disease
IP	prostaglandin I receptor
I/R	ischemia reperfusion
K-ATP	ATP-sensitive potassium channels
KCL	potassium chloride
LDH	lactate dehydrogenase
L-NMMA	N^G-mono-methyl-L-arginine
LSD	least significant difference
LT	leukotrienes
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTC_4	leukotrienes C_4
LTD_4	leukotrienes D_4
LTE_4	leukotrienes E_4
Mn-SOD	manganese superoxide dismutase
NAD	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate

NADP	nicotinamide adenine dinucleotide phosphate
NANC	non-adrenergic non-cholinergic
NH_2Cl	monochloroamine
NO	nitric oxide
NO_2	nitrogen dioxide
NO_3	nitrate
NOS	nitric oxide synthase
O_2	oxygen molecule
$O_2^{\cdot -}$	superoxide anion
O_2^{-2}	hydrogen peroxide dianion
$^1O_2$	singlet oxygen
$^1O_2\Delta g$	delta singlet oxygen
$^1O_2\Sigma g+$	sigma singlet oxygen
OFR	oxygen free radicals
$\cdot OH$	hydroxyl radical
$ONOO^-$	peroxynitrite anion
ONOOH	peroxynitrous acid
P	probability
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_2\alpha$	prostaglandin $F_2\alpha$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PMNL	polymorphonuclear leukocyte
PUFA	polyunsaturated fatty acids
$ROO\cdot$	peroxy radical
ROOH	hydroperoxide
SEM	standard error of mean
SH	sulfhydryl

SOD	superoxide dismutase
SR	sarcoplasmic reticulum
TK	tachykinins
TXA_2	thromboxane A_2
VIP	vasoactive intestinal peptide
Vit-C	vitamin-C
$Vit\text{-}C^{\cdot -}$	semidehydroascorbate radical anion
Vit-E	vitamin-E
X	xanthine
XO	xanthine oxidase
XOD-HPX	xanthine oxidase-hypoxanthine
X/XO	xanthine/ xanthine oxidase

1.0 LITERATURE REVIEW

1.1 General Characteristics of Oxygen Free Radicals

Electrons in an atom or molecule occupy spatial volume elements, called orbitals, each of which can contain two electrons under certain restrictions. A free radical may be defined as any species that has one or more unpaired electrons. Free radical reactivity is accounted for by the strong tendency of the unpaired electron to interact with other electrons to form an electron pair and thus a chemical bond. This broad definition includes the hydrogen atom, most transition metals and the oxygen molecule itself (Halliwell and Gutteridge, 1984b).

Oxygen (O_2) is considered to be essential and beneficial to all aerobic organisms. About one fourth of 1 kg of oxygen per day is used for respiration by a human. While the ultimate fate of this oxygen is conversion into water and carbon dioxide, in the process it undergoes many enzymatic reactions, involving free radical formation (Green and Hill, 1984). Oxygen supplied at concentrations greater than those in normal air has long been known to damage plants, animals and aerobic bacteria. In 1954, Gershman and Gilbert proposed that many of the damaging effects of oxygen could be attributed to the formation of oxygen radicals (for a review

see Gilbert, 1981). Reactive oxygen species include not only oxygen-centered radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl (.OH) radicals, but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$).

1.1.1 Superoxide Anion Radical

The addition of a single electron to the oxygen molecule results in the formation of the superoxide anion ($O_2^{\cdot -}$), which is formed in almost all aerobic cells (Halliwell and Gutteridge, 1984b). Superoxide may also be protonated to form $HO_2^{\cdot}$, the perhydroxyl radical; however, only about 1% of the protonated form of $O_2^{\cdot -}$ will exist at neutral pH (Kukreja and Hess, 1992), and at a physiologic pH of 7.4, the superoxide anion will be predominant.

A variety of enzyme systems catalyze the univalent reduction of molecular oxygen to superoxide anion radical. Examples include xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase, flavin dehydrogenase, and peroxidase. The proportion of $O_2^{\cdot -}$ released from the active site of these enzymes depends on pH, oxygen concentration; and substrate concentration (Freeman and Crapo, 1982).

The autooxidation of a large group of compounds including catecholamines, flavins, thiols and hydroquinones also involves the release of $O_2^{\cdot -}$. Another important source of $O_2^{\cdot -}$ is the mitochondrial electron transport system. Free

radicals are continuously formed as normal byproducts of cellular metabolism. The mitochondrial electron transport system links the production of adenosine triphosphate (ATP) to the controlled tetravalent reduction of molecular oxygen to water (Freeman and Crapo, 1982). Normally about 1-5% of oxygen that is reduced to water within the mitochondria leaks out from the tetravalent reduction pathway and undergoes univalent reduction (Freeman and Crapo, 1982). The intra-mitochondrial concentration of $O_2^{\cdot -}$ has been estimated to be 8×10^{-12} M (Freeman and Crapo, 1982).

Superoxide anion is also produced by the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase system present on the surface of inflammatory cells. One important source is the "respiratory burst" of phagocytic cells when they contact foreign particles or immune complexes (Halliwell, 1982). Phagocytic cells known to produce $O_2^{\cdot -}$ include neutrophils, monocytes, macrophages and eosinophils.

Superoxide is less reactive compared to the hydroxyl radical; however, it has the potential to react with a variety of biological substrates in aqueous media as either a reductant or oxidant (Singh, 1982). As a reductant, superoxide anion loses an electron and is oxidized back to molecular oxygen. The biological substrates reported to be reduced by it include ferricytochrome c and quinones, as well as the transition metal complexes. When superoxide anion acts as an oxidant, it gains an electron from the substrate and is

reduced to hydrogen peroxide. Examples include the oxidation of ascorbic acid, α-tocopherol, catecholamines, hemoproteins and thiols (Fridovich, 1983).

A number of biological targets can be attacked by superoxide radical. Superoxide-dependent initiation of lipid peroxidation is generally thought to occur via an iron-catalyzed Haber-Weiss mechanism. The role of $O_2^{\cdot -}$ in this process is 2-fold: generation of H_2O_2 by non-enzymatic dismutation and reduction of ferric iron to the ferrous form (Thomas et al., 1985). Superoxide may attack enzymes containing iron-sulfur clusters, or inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Halliwell, 1993). It has been reported that superoxide can also react with nitric oxide (NO), a free radical produced by several cell types, to give peroxynitrite (Halliwell, 1993). Peroxynitrite decomposes to form hydroxyl radical.

1.1.2 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) may be formed as a result of two types of reactions: (1) divalent reduction of O_2 which is carried out by a group of enzymes such as D-amino acid, and glycolate oxidases, and (2) univalent reduction of O_2 to $O_2^{\cdot -}$ which occurs during the aerobic oxidation of substrates by certain enzymes such as xanthine oxidase (Fridovich, 1970), or autooxidation of various compounds with the

subsequent dismutation of $O_2^{\cdot -}$ to H_2O_2.

The divalent reduction product of O_2 is the hydrogen peroxide dianion (O_2^{-2}). This species is not an oxygen radical because all electrons are now paired; however, any O_2^{-2} formed at physiological pH will immediately be protonated to give hydrogen peroxide. Dismutation of $O_2^{\cdot -}$ occurs in two steps. Firstly, $O_2^{\cdot -}$ combines with a proton to form the hydroperoxyl radical ($HO_2^{\cdot}$). Secondly, this radical reacts with another $O_2^{\cdot -}$ and a hydrogen atom, producing H_2O_2. The spontaneous dismutation of $O_2^{\cdot -}$ occurs at a rate of $1 \times 10^5\ M^{-1}\ S^{-1}$.

$$O_2^{\cdot -} + H^+ \text{ ----------> } HO_2^{\cdot} \qquad [1.1]$$

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \text{ ----------> } H_2O_2 + O_2 \qquad [1.2]$$

Also, two hydroxyl radicals can join their unpaired electrons and form an oxygen-oxygen covalent bond, giving H_2O_2:

$$^{\cdot}OH + {}^{\cdot}OH \text{ ----------> } H_2O_2 \qquad [1.3]$$

Peroxisomes are important sources of cellular H_2O_2 because they contain a high concentration of oxidases (D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, and fatty acyl-CoA oxidase), none of which has been shown to generate $O_2^{\cdot -}$ as an intermediate precursor of H_2O_2 (Freeman and Crapo, 1982). The proportion of peroxisomal H_2O_2 that can diffuse into the cytoplasm ranges from two percent to a measured 11

to 42 percent (Freeman and Crapo, 1982).

Hydrogen peroxide crosses cell membranes easily and can enter into the extracellular environment, which contains fewer antioxidant defense mechanisms as compared to that of the intracellular space (Halliwell, 1993). H_2O_2 is a strong oxidant but reacts only slowly with most organic substances. However, it can react with transition metal ions and their inorganic or organic complexes at rapid rates to generate powerful oxidants. Hydrogen peroxide can also deplete cellular glutathione (GSH) and reduced nicotinamides during glutathione peroxidase-catalyzed H_2O_2 reduction and serve as a precursor for hydroxyl radical production (Freeman and Crapo, 1982).

Inactivating of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, inhibition of the cytoplasmic CuZn-SOD, the generation of myeloperoxidase-catalyzed hypohalous acids, the activation and inactivation of arachidonic acid metabolism, bradykinin release, the synthesis of platelet activating factor, inhibition of lymphocyte proliferation and an increase in intracellular free calcium are another processes that can be influenced by H_2O_2 (Asbeck, 1990; Halliwell, 1993).

On the whole, $O_2^{\cdot -}$ and H_2O_2 have limited chemical reactivity; thus, the major determinant of the actual toxicity of $O_2^{\cdot -}$ and H_2O_2 to cells may well be the availability and location of metal ion catalysts of hydroxyl radical

formation (Halliwell, 1993).

1.1.3 Hydroxyl Radical

Hydroxyl radical ($\cdot OH$) is produced in living organisms by at least two mechanisms: homolytic fission of water due to background exposure to ionizing radiation, and reaction of transition metal ions with H_2O_2 (Halliwell, 1993).

Homolytic fission of the O-O bond in H_2O_2 produces two hydroxyl radicals. Homolysis can be achieved by heat or ionizing radiation. A simple mixture of H_2O_2 and an iron (II) salt will also produces the $\cdot OH$ radical , as first observed by Fenton.

$$Fe^{2+} + H_2O_2 \text{ ----------> } Fe^{3+} + \cdot OH + OH^- \qquad [1.4]$$

When the iron concentration is limiting, the reduction of the Fe^{3+} formed is required for the continued formation of hydroxyl radical. This can be accomplished by the superoxide anion ($O_2^{\cdot -}$).

$$Fe^{3+} + O_2^{\cdot -} \text{ ----------> } Fe^{2+} + O_2 \qquad [1.5]$$

$$Fe^{2+} + H_2O_2 \text{ ----------> } Fe^{3+} + OH^- + \cdot OH \qquad [1.6]$$

$$\text{Net: } O_2^{\cdot -} + H_2O_2 \text{ -------> } O_2 + OH^- + \cdot OH \qquad [1.7]$$

This sequence of reactions has been termed the iron-catalyzed Haber-Weiss reaction or the superoxide-driven Fenton

mechanism (Haber and Weiss, 1934). In fact, the iron-catalyzed Haber-Weiss reaction involves the direct reduction of hydrogen peroxide by the superoxide anion. However, under most physiological conditions, the Haber-Weiss reaction occurs very slowly, and does not occur to any significant extent in the absence of iron or other redox-active transition metals (McCord and Omar, 1993). However, some investigators suggested that even the rate constant for the Fenton reaction is too low, calculations based on rate constants suggest that $\cdot OH$ may be generated at the rate of approximately 50/cell/s, which could have enormous biological consequences (Stohs and Bagchi, 1995).

Another pathway for the generation of $\cdot OH$ has been described by Halliwell, (1993). It is proposed that the $\cdot OH$ is generated during an iron-independent reaction involving the interaction of $O_2^{\cdot -}$ and nitric oxide (NO). The proposed reaction is as follows:

$$NO + O_2^{\cdot -} \text{-------} > ONOO^- \quad [1.8]$$

$$ONOO^- \text{----------} > ONOOH \quad [1.9]$$

$$ONOOH \text{---------} > \cdot OH + \cdot NO_2 \,/\, NO_3 \quad [1.10]$$

Where:

$ONOO^-$ = peroxynitrite anion

ONOOH = peroxynitrous acid

$\cdot NO_2$ = nitrogen dioxide

NO_3 = nitrate

Investigation into the metabolism of arachidonic acid by cyclooxygenase and lipoxygenase has revealed that intermediate peroxy compounds and hydroxyl radicals are also produced in these reactions. The hydroxyl radical results from the peroxidative conversion of endoperoxide PGG_2 to PGH_2 (McCord and Omar, 1993).

Formation of ·OH may present the cell with numerous problems. The hydroxyl radical is an extremely powerful oxidant. It is unstable and short-lived (10^{-6} s), with a diffusion radius of 2.3 nm (Pryor, 1982). Because of its reactivity, ·OH reacts with almost all biological substrates at biomolecular rate constants ranging from 10^7 -10^{10} M^{-1} sec^{-1} (Dorfman and Adams, 1973). Indeed, the extremely reactive nature of the ·OH suggests that it will only mediate direct effects close to its site of generation or must rely on the formation of less reactive secondary species to exert long-range effects.

Hydroxyl radical toxicity may be mediated by the products of ·OH-induced interactions with lipids or other cellular components. Hydroxyl radical will oxidize sulfhydryl (SH) compounds that may result in inactivation of essential enzymes or membrane transport proteins. The radical will also react with DNA to cause "nicks" or other abnormalities (Burger, et al., 1980). Membrane-associated polyunsaturated fatty acids are also readily attacked by ·OH, resulting in lipid peroxidation (Aust and Svingen, 1982). This oxidant

(·OH) also regulates prostaglandin and thromboxane production by multiple mechanisms, which include deactivation of cyclooxygenase, peroxidase and prostacyclin synthase (McCord and Omar, 1993).

It should be noted that the cellular mechanisms that have evolved avoid the formation of the trivalent reduction product of oxygen, ·OH, and only with the breakdown and overwhelming of these intracellular mechanisms would ·OH be formed intracellularly.

1.1.4 Singlet Oxygen

Singlet oxygen ($^1O_2$) by definition is not a radical species. Rather, it is an electronically excited state of oxygen that results from the promotion of an electron to higher energy orbitals (Halliwell and Gutteridge, 1984b). Two singlet states of O_2 exist, namely delta $^1O_2$ and sigma $^1O_2$. Delta $^1O_2$ ($^1O_2\Delta g$), the most important in biological forms, is formed when the unpaired electrons of molecular oxygen are paired and occupy the same orbital, so that it has no unpaired electrons and is therefore not a radical. Sigma $^1O_2$ ($^1O_2\Sigma g+$) exists when the electrons of molecular oxygen inhabit different energy orbitals and spin in opposite directions. The short-lived sigma singlet usually decays to the delta state before it has time to react with anything (Halliwell and Gutteridge, 1984b).

Singlet oxygen is generated enzymatically by the halide-

mediated H_2O_2 decomposition by lactoperoxidase, chloroperoxidase and myeloperoxidase. Furthermore, $^1O_2$ is generated by the reaction of the following enzymes: xanthine oxidase, lipooxygenase, prostaglandin-endoperoxide synthase, as well as cytochrome P-450-catalyzed heterolytic cleavage of hydroperoxides (Kukreja and Hess, 1992). It is also generated as a result of the self-reaction of lipid peroxy radicals in the termination step of lipid peroxidation. Also, interaction of $O_2^{\cdot -}$ with H_2O_2 via the non-enzymatic dismutation reaction results in $^1O_2$ formation:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \text{----------} > H_2O_2 + O_2 \qquad [1.11]$$

$$H_2O_2 + O_2^{\cdot -} \text{----------} > {\cdot}OH + OH^- + {}^1O_2 \qquad [1.12]$$

Although $^1O_2$ has a short half life (10^{-5} s), this is sufficient to allow localized diffusion and interaction with membrane protein or lipid. Singlet oxygen reacts with molecules chemically or by the transfer of its energy. Like oxygen free radicals, however, if singlet oxygen is released in biological systems, it is capable of rapidly oxidizing many molecules, including membrane polyunsaturated fatty acids. It can also damage DNA, can lead to the inactivation of many enzymes, and can oxidize mitochondrial components (Kukreja and Hess, 1992).

1.2 Iron and Oxygen Free Radicals

Following its ingestion, iron is either oxidized and stored in the iron storage protein ferritin, or become associated with the iron transport protein transferrin in the blood stream (Halliwell and Gutteridge, 1984a; Halliwell and Gutteridge, 1986). Dietary iron, which is absorbed almost exclusively in the ferrous state, is oxidized to the ferric state by xanthine oxidase in the mucosal cells (Topham et al., 1982). The enzyme xanthine oxidase therefore promotes the incorporation of iron into transferrin, which mediates the transcellular transport of iron in the mucosal cell. Iron is the most important catalyst of oxidative processes, due to the fact that its concentration in animal tissues is much higher than that of other transition metals (Asbeck, 1990). For iron to facilitate the formation of reactive oxygen species via the Fenton reaction, the iron must be in a free or catalytically active form; however, most of the iron is complexed, and little free iron actually exists in nature.

Generally, the cell is adequately protected against the damaging effects of iron by its effective complexing with transferrin, haemosidrin or ferritin (Bothwell and Charlton, 1982), and by compartmentalizing these pools of iron away from susceptible membrane sites. Iron bound to these proteins is unable to catalyze $\cdot OH$ formation (Halliwell and Gutteridge, 1986). However, $O_2^{\cdot -}$ was found to be able to mobilize iron from ferritin, making iron available to

catalyze ·OH formation (Biemond et al., 1986). Xanthine oxidase may also promote the release of iron from ferritin by direct reduction of the ferric iron (Thomas et al., 1985).

Within cells, there is also some form of low-molecular mass transit pool of iron to promote the production of ·OH and lipid peroxidation (Bacon and Tavill, 1984). The small pool of non-protein-bound iron moving between transferrin, cell cytoplasm, mitochondria and ferritin could provide iron for the Fenton reaction. Thomas et al., (1985) suggested a novel role for $O_2^{\cdot -}$ in promoting toxicity, that is, the reductive release of iron from ferritin, thereby potentially increasing the low molecular weight iron pool capable of undergoing redox reactions leading to the formation of stronger oxidants. However, enzymes such as superoxide dismutase, glutathione peroxidase and catalase could be the first line of defense, removing $O_2^{\cdot -}$ and H_2O_2 before they get anywhere near the iron promoters.

In cells, iron can also be present bound to compounds such as ATP, GTP and citrate. These iron-chelate complexes are very well able to catalyze ·OH formation, apparently even better than free iron. This effect may be due to a much faster reduction of the iron-chelate as compared to free iron. Furthermore, iron-chelate complexes are much more soluble than free iron, which forms a precipitate of $Fe(H_2O)_6^{3+}$. Thus, with iron chelators present, the likelihood of ·OH formation from $O_2^{\cdot -}$ and H_2O_2 is very much increased

(Graf et al., 1984).

A role for intracellular iron and hydroxyl radicals has been implicated in H_2O_2-mediated bacterial killing (Repine et al., 1981) and neutrophil-mediated endothelial cell injury (Gannon et al., 1987). Extracellularly generated H_2O_2 can enter the cell and interact with non-ferritin iron to produce the cytotoxic $\cdot OH$ (Kvietys et al.,1989). Furthermore, Gannon et al., (1987) have demonstrated that neutrophil-mediated endothelial cell injury can be prevented by pre-treatment of the endothelial cells with deferoxamine, an iron chelator. Pre-treatment with deferoxamine also reduced the oxy-radical-induced extravasation of red blood cells form rat gastric, indicating that iron plays an important role in mediating the oxy-radical-induces responses (Smith et al., 1987).

1.3 Protective Mechanisms Against Oxygen Free Radicals

Eukaryotic cells are not defenseless in the face of oxidant attack. Complex sets of protective mechanisms have evolved in cells that are designed to prevent, limit or repair oxidative damage. This defense system consists of enzymatic and nonenzymatic factors.

1.3.1 Enzymatic Antioxidant Defenses

The enzymatic antioxidant defenses consist of enzymes able to detoxify $O_2^{\cdot -}$, H_2O_2 and organic peroxides. None of the known enzymatic antioxidants are able to detoxify $\cdot OH$ or $^1O_2$.

Because of the strong oxidizing potential of $\cdot OH$ and $^1O_2$ it is unlikely that any enzyme could detoxify these species before undergoing oxidative damage itself.

1.3.1.1 Superoxide Dismutase

Superoxide dismutases (SOD) are a family of metalloproteins that catalyze the dismutation of $O_2^{\cdot -}$ to H_2O_2 (Fridovich, 1983);

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad [1.13]$$

SOD-mediated rates of $O_2^{\cdot -}$ dismutation are enormously faster than the spontaneous reaction. The spontaneous dismutation of $O_2^{\cdot -}$ occurs at a rate of $1 \times 10^5\ M^{-1}\ S^{-1}$; however, the rate constant for the reaction catalyzed by the enzyme SOD is $1 \times 10^9\ M^{-1}\ S^{-1}$ (Fridovich, 1983). Cells are capable of increasing synthesis of SOD in response to hyperoxidant stress (Fridovich, 1983). In the presence of SOD, $O_2^{\cdot -}$ does not react with potential substrates (other than itself or its protonated form), or drive $O_2^{\cdot -}$-dependent Haber-Weiss reactions to form $\cdot OH$ or $^1O_2$. However, it should be noted that $O_2^{\cdot -}$ could escape enzymatic dismutation if it is generated near or at a site inaccessible to SOD (Fridovich, 1983).

In mammalian cells, three different kinds of SOD's have been discovered (Fridovich, 1978; Fridovich, 1988). The copper-zinc enzyme (CuZn-SOD) is found in the cytosol and the

intermembrane space in mitochondria. Manganese-SOD (Mn-SOD) has been found in the matrix space of mitochondria and, in small amounts, in the cytoplasmic matrix of rat liver parenchymal cells, and extracellular SOD (EC-SOD) which is a major SOD isoenzyme in extracellular fluid, but also occurs in tissues (Marklund, 1984). EC-SOD may normally exist bound to the surfaces of vascular endothelial cells, from which it sometimes detaches off to leave a low activity in the plasma. It should be noted that in the extracellular space, the concentration of $O_2^{\cdot -}$ is not as strictly controlled, because there are traces of CuZn-SOD, Mn-SOD, and EC-SOD (Marklund, 1982; Marklund, 1984).

1.3.1.2 Catalase and Glutathione Peroxidase

Hydrogen peroxide is reduced by either the heme-enzyme, catalase or the selenoenzyme, glutathione peroxidase (GSH-Px) (Chance et al., 1979). Catalase is detectable in almost all mammalian cells although its concentration can vary significantly among various tissue types (Ishikawa and Sies, 1984). It may be found in the cytosol, but in many tissues it is localized in peroxisomes and microperoxisomes (Chance et al., 1979). Catalase is an enzyme which catalyzes the decomposition of hydrogen peroxide to oxygen and water:

$$AH_2 + H_2O_2 \xrightarrow{\text{Catalase}} A + 2H_2O \qquad [1.14]$$

(peroxidatic reaction)

Where: AH_2 = Electron donor

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O \quad [1.15]$$

(catalytic reaction)

Glutathione peroxidase can reduce H_2O_2 by catalyzing its reaction with reduced glutathione (GSH) to form oxidized glutathione disulfide (GSSG) and water:

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} 2H_2O + GSSG \quad [1.16]$$

GSH-Px also catalyzes the removal of lipid peroxides (Gunzler et al., 1972). Sixty to 75% of the enzyme activity is found in the cytoplasm of eukaryotic cells and 25-40% in the mitochondria (Michiels et al., 1994). In order for this enzyme to continue to reduce a flux of H_2O_2, the GSSG must be reduced back to GSH by the NADPH-dependent enzyme, GSSG reductase.

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2GSH + NADP^+ \quad [1.17]$$

Many authors have claimed the superiority of GSH peroxidase in removing H_2O_2 at low steady state levels, while catalase

was held primarily responsible for the metabolism of high amounts of H_2O_2. Because catalase affinity for H_2O_2 is low, it may need high H_2O_2 concentrations to work efficiently (Flohe, 1982).

As opposed to the intracellular compartment, the extracellular space does not appear to be well equipped with enzymatic systems designed to reduce H_2O_2. Because extracellular H_2O_2 could be rapidly metabolized by circulating erythrocytes (H_2O_2 rapidly crosses the erythrocyte membrane where it would come in contact with catalase-GSH peroxidase) it has been suggested that these cells could represent a potential defense system in the extracellular space (Test and Weiss, 1984).

There is no enzyme that metabolizes $\cdot OH$ and singlet oxygen, but the body does have non-enzymatic hydroxyl radical scavengers. Two six-carbon compounds, glucose and mannitol, are hydroxyl radical scavengers with similar rate constants (Halliwell and Gutteridge, 1986).

1.3.2 Nonenzymatic Antioxidant Defenses

Nonenzymatic antioxidant defenses consist of molecules that react with activated oxygen species and in this way block the propagation of free radical chain reactions. The natural non-enzymatic antioxidants can be divided into membrane-bound and water soluble antioxidants, according to the site of their antioxidative action. Vitamin E (Vit-E) and

beta-caroten are the most important membrane-bound antioxidants. Ascorbic acid and GSH belong to the group of water soluble antioxidants. Owing to the reaction of Vit-E with ascorbic acid, an interrelationship exists between the antioxidant defense in the lipophilic and the hydrophilic phases (Freeman and Crapo, 1982).

1.3.2.1 Vitamin E

Vitamin E (α-Tocopherol), a lipid soluble antioxidant, intercalates within all cellular and organelle membranes, particularly those of the endoplasmic reticulum and mitochondria. This antioxidant converts radicals to less reactive forms, thus protecting these membranes against lipid peroxidation. The main function of vitamin E is to rapidly reduce ($K= 10^6$-10^8 M^{-1} S^{-1}) polyunsaturated lipid peroxide free radicals by a nonenzymatic reaction:

$$\text{vit-E} + \text{LOO}\cdot \longrightarrow \text{vit-E}\cdot + \text{LOOH} \qquad [1.18]$$

It is assumed that vitamin E radical may be repaired by reaction with an H-donor such as ascorbic acid.

Vitamin E can scavenge $O_2^{\cdot -}$, $\cdot OH$, singlet oxygen and lipid peroxy radicals (Freeman and Crapo, 1982; Prasad and Kalra, 1993). The reactivity of vitamin E with peroxylipid radicals may be on the order of 10^4 times that of the reaction of these radicals with polyunsaturated fatty acids (Horton and

Fairhaust, 1987). Tocopherols are probably the major chain-breaking antioxidants in human membranes.

1.3.2.2 Beta Carotene

Beta carotene (β-Carotene), a precursor of Vitamin A, can scavenge singlet oxygen and dissipate its energy without producing cellular injury. It is an effective lipid-soluble quencher of $^1O_2$. It exhibits efficient radical-trapping antioxidant activity only at partial pressures of oxygen which are significantly lower than in normal air and similar to the partial pressures found in most tissues under physiological conditions (Horton and Fairhaust, 1987).

1.3.2.3 Glutathione

Glutathione (GSH) is a water-soluble antioxidant and is found in the cytosol. It is a major component in the enzymatic removal of H_2O_2 or organic peroxides (Kosower and Kosower, 1974). However, glutathione can also react with $O_2^{\cdot -}$, $^{\cdot}OH$, and $^1O_2$ non-enzymatically (Kosower and Kosower, 1974):

$$O_2^{\cdot -} + H^+ + GSH \text{ ------> } GS^{\cdot} + H_2O_2 \qquad [1.19]$$

$$^{\cdot}OH + GSH \text{ --------> } GS^{\cdot} + H_2O \qquad [1.20]$$

$$^1O_2 + GSH \text{ -------> } GS_{ox} \text{ (cysteic acid)} \qquad [1.21]$$

The thiyl radical of glutathione ($GS^{\cdot}$), formed during the reaction, is relatively stable and supposedly causes no

further damage.

1.3.2.4 Vitamin C

The aqueous phase of plasma contains a wide range of molecules with antioxidant properties. One of these is Vitamin C (ascorbic acid), an effective scavenger of $O_2^{\cdot -}$, $^{\cdot}OH$ and $^1O_2$. Ascorbate can also act as a secondary antioxidant by reacting with Vitamin E radicals to be generate Vitamin E. Vitamin C may have intrinsic pro-oxidant effects as well (Groot, 1994). For instance, it can reduce Fe^{3+} to yield Fe^{2+} and the semidehydroascorbate radical anion (Vit $C^{\cdot -}$):

$$Fe^{3+} + \text{Vit C} \text{ --------} > Fe^{2+} + \text{Vit } C^{\cdot -} + 2H^{+} \qquad [1.22]$$

Then, Fe^{2+} may lead to the formation of the very reactive oxygen species, hydroxyl radical.

1.3.2.5 Other Biological Compounds with Antioxidant Properties

Other aqueous scavengers, including transferrin, urate, ceruloplasmin and even albumin, all appear to act as primary scavengers, reacting directly with reactive oxygen metabolites to produce more stable compounds. Transferrin, like ferritin, binds iron, thereby preventing its acting as a catalyst to generate more toxic secondary species. Uric acid can react as an antioxidant, both by binding iron and

copper ions, and by directly scavenging of singlet oxygen and peroxy radicals (Halliwell and Gutteridge, 1990a, Halliwell and Gutteridge, 1990b). The plasma copper-containing protein ceruloplasmin plays an important role in iron metabolism. Antioxidant activity of ceruloplasmin can be mainly ascribed to its ferroxidase activity, which effectively inhibits ferrous ion-stimulated lipid peroxidation and ferrous ion-dependent formation of ·OH (Halliwell and Gutteridge, 1990a; Halliwell and Gutteridge, 1990b). It also reacts very slowly with $O_2^{\cdot -}$ (Goldstein et al., 1979) and H_2O_2 (Halliwell and Gutteridge, 1990a; Halliwell and Gutteridge, 1990b). Albumin can bind copper ions, and inhibits copper ion-dependent lipid peroxidation and ·OH radical formation.

Bilirubin, glucose, amino acids such as histidine, tryptophan, and methionine are other extracellular components that have antioxidant activity.

1.4 Oxygen Free Radical-Induced Injury

1.4.1 Extracellular Space

The extracellular components of tissue at highest risk to oxidizing species are collagen and hyaluronic acid (Freeman and Crapo, 1982). The inhibition of collagen gelation by $O_2^{\cdot -}$ results in the loss of cartilage integrity, whereas the polymerization of hyaluronic acid by $O_2^{\cdot -}$ can change synovial fluid viscosity (Fridovich, 1978; Freeman and Crapo, 1982).

1.4.2 Cellular Elements at Risk from Oxygen Free Radicals

The membranes of mammalian cells contain large amounts of polyunsaturated fatty acids which can undergo peroxidative injury (Mead, 1976). The process of degradation of polyunsaturated phospholipid membranes is commonly referred to as lipid peroxidation. Lipid peroxidative chain reactions of both liposomal and cellular membranes can be induced by enzymatically generated free radicals (Kellogg and Fridovich, 1975). Hydroxyl radical and singlet oxygen can react with the unsaturated lipids of membranes resulting in the generation of lipid peroxide radicals (ROO.), lipid hydroperoxides (ROOH) and fragmentation products such as malondialdehyde, which are all toxic (Kellogg and Fridovich, 1975). These events induce profound changes in the structure of biological membranes, resulting in the loss of membrane fluidity, breakdown of membrane secretory functions and transmembrane ionic gradients (Southorn and Powis, 1986; Southorn and Powis, 1988). It has also been suggested that a product of radical generation can alter phospholipase activity, which would result in arachidonic acid release and subsequent formation of stable prostaglandins and various endoperoxides (Del Maestro, 1979).

All cellular components can react with OFRs at the level of unsaturated bonds and thiol groups. Consequently, proteins containing amino acid with unsaturated bonds, as well as sulfur, can be easily attacked by oxygen free radicals and

undergo free radical-mediated amino acid modification. In proteins, some amino acids are very sensitive to such attack, including tryptophan, tyrosine, phenylalanine, histidine, methionine and cysteine, inducing enzymatic activity alterations or changes in conformation (Freeman and Crapo, 1982). For example, enzymes such as glyceraldehyde-3-phosphate dehydrogenase, which depends upon these amino acids for its reactivity, are inhibited in the presence of OFRs. Inhibition of this enzyme will lead to a decrease in the production of ATP.

Hydroxyl radical can also induce protein cross-linking and cleave amino acid bonds, leading to fragmentation of the macromolecules. Peptide bonds or amino acids such as proline and lysine can be affected by hydroxyl radicals (Trelstad et al., 1981).

Nucleic acids are another target for free radical attack, generating DNA strand breaks or base modifications, leading to point mutations. Hydroxyl radical alone has been implicated in causing more than 80 percent of radiation-induced cell death (Freeman and Crapo, 1982). The damage to the nucleic acids leads to the inhibition of nucleotide and protein synthesis which are essential for ATP supplies and enzymatic production, respectively.

It is now thought that not only lipid peroxidation but also alteration of nucleic acids and proteins by free radicals are responsible for cell death during oxidative

stress (Michiels et al., 1994).

1.5 Normal Physiology of Small Intestinal Motility

The small intestine, the largest segment of the gastrointestinal system, accounts for about three fourths of the length of the human gastrointestinal tract. The structure of the gastrointestinal tract varies from region to region, but there are common features in the overall organization of the tissue. The general components of the layered structure of the wall of the gastrointestinal tract are: the mucosa, submucosa, muscularis externa and adventia (Berne and Levy, 1993; McKay and Perdue, 1993; Otterson and Sarr, 1993; Perdue and McKay, 1994).

The mucosa consists of an epithelium, the lamina propria, and the muscularis mucosa. The nature of the epithelium varies from one part of the digestive tract to another. The epithelium is a layer of single cells lining the lumen that extends over the mucosal surface, covering the villi of the small intestine and extending down into the crypts. This epithelium forms the interface between the external environment (i.e. the intestinal lumen) and the interstitial space. The main cells in the epithelium are the transporting enterocytes, but other cell types are also present in large numbers. These include goblet cells, various enteroendocrine cells, intraepithelial leukocytes, M-cells, and, in the crypt, paneth cells. The epithelial cells rest on

a basement membrane. The basement membrane separating the epithelium from the lamina propria is a product of both epithelial cells and fibroblasts. Functionally, the intestinal epithelium acts as a selective barrier to prevent the entry of antigenic or infectious material into the body. The lamina propria consists largely of loose connective tissue containing collagen and elastin fibrils, and a variety of immune cells (lymphocytes, plasma cells, macrophages, mast cells, eosinophils and neutrophils). It is also rich in several types of glands and contains lymph nodules and capillaries. The muscularis mucosa is the thin innermost layer of intestinal smooth muscle. Contractions of the muscularis mucosa force the mucosa into folds and ridges.

The submucosa consists largely of loose connective tissue with collagen and elastin fibrils. The larger blood vessels of the intestinal wall travel in the submucosa. The muscularis externa consists of two substantial layers of smooth muscle cells: an inner circular layer and an outer longitudinal layer. The circular layer is three to five times thicker than the longitudinal layer, and it is mainly responsible for mixing and propulsion in the gastrointestinal tract (GI-tract). The circular layer comprises an inner dense circular layer and a thicker outer circular layer.

The serosa, or adventia, is the outermost layer and consists mainly of connective tissue covered with a layer of squamous mesothelial cells (Berne and Levy, 1993; McKay and

Perdue, 1993; Otterson and Sarr, 1993; Perdue and McKay, 1994).

The small bowel accomplishes the absorption of ingested nutrients by utilizing specific spatial and temporal patterns of contractile activity. These contractions mix and propel ingested food forward at a rate that facilitates digestion and absorption, they keep the gastrointestinal tract free of debris, secretions, and bacteria between meals, and they propel intestinal contents rapidly over great distances independently of digestion and absorption during special situations (such as vomiting and mass movement), which may serve to protect the organism.

These motor functions of the small intestine are accomplished by both the circular and the longitudinal layers of smooth muscle in the gut. The various spatial and temporal patterns of contractile activity are under myogenic, neural and chemical control. Each of these mechanisms is subject to modulation by the immune system, which can influence the excitability of smooth muscle, peripheral, enteric and central nerves (Berne and Levy, 1993; McKay and Perdue, 1993; Otterson and Sarr, 1993; Perdue and McKay, 1994).

1.5.1 Myogenic Control

"Myogenic control" refers to the electrical activity generated by the smooth muscle of the gut. Electrical control activity (ECA; also called slow wave activity, basic

electrical rhythm, or pace setter potential) is the omnipresent rhythmic depolarization of the cell membranes of smooth muscle of the small intestine. Slow waves are generated by specialized interstitial cells. A thin layer of interstitial cells is present at the border between the circular and longitudinal smooth muscle layer; another thin layer of interstitial cells is present between the inner dense circular and the outer circular smooth muscle. Slow wave that is generated in the interstitial cells is rapidly conducted through all the cells of the muscularis externa via the gap junctions that interconnect the smooth muscle cells.

Normally, the resting membrane potential of intestinal smooth muscle cells is -50 to -70 mV relative to the extracellular fluid. With neural or chemical stimulation, membrane depolarization exceeds an excitation threshold, and a contraction results. While the patterns of contractile activity are ultimately under myogenic control, whether or not a contraction will occur at any given site depends on local neurochemical stimulation. This requirement allows for modulation of contractile activity by regulatory mechanisms outside the gut (Berne and Levy, 1993; Otterson and Sarr, 1993).

1.5.2 Neural Control

The small intestine smooth muscle is influenced by both extrinsic autonomic nerves (parasympathetic and sympathetic)

from the CNS and the intrinsic neurons of the enteric nervous system.

1.5.2.1 Extrinsic Neural Control

Extrinsic neural control is provided by parasympathetic and sympathetic nervous system. Parasympathetic innervation of the gastrointestinal tract down to the level of the transverse colon is provided by branches of the vagus nerve. The reminder of the colon receives parasympathetic fibers from the pelvic nerves by way of the hypogastric plexus. The small intestine derives its parasympathetic innervation through the vagus. The vagus contains both efferent and afferent fibers. Efferent motor fibers arise from the dorsal motor nucleus in the region of the fourth ventricle. Vagal afferents detect both mechanical and chemical stimulation of the small intestine and relay this information centrally for processing. Although the precise role of the vagus is unknown, electrical stimulation of the thoracic vagus will induce contractions of the upper small intestine.

The sympathetic innervation of the small intestine arises from the thoracic and lumbar spinal nerves (generally T5 through L3). These nerves pass through the para-vertebral ganglia and form the splanchnic nerves, which go to the pre-vertebral ganglia - the celiac, superior mesenteric, and inferior mesenteric. Most of the sympathetic fibers terminate in the submucosal and myenteric plexuses. Some sympathetic

fibers innervate blood vessels of the GI-tract, causing vasoconstriction. Other sympathetic fibers innervate glandular structures in the wall of the gut. Relatively few of the sympathetic fibers terminate in the muscularis externa. As with vagus, sympathetic innervation supplies a modulatory influence on contractile activity. Stimulation of the sympathetic input to the GI-tract inhibits motor activity of the muscularis externa, and has an inhibitory effect on synaptic transmission in the enteric plexuses (Berne and Levy, 1993; Otterson and Sarr, 1993).

1.5.2.2 Enteric Nervous System Control

The enteric nervous system (ENS), the "minibrain" of the gut, consists of a coordinated network of neurons (about 10^8 in number) having their cell bodies within the bowel wall. These neurons have extensive connections with each other, as well as with extrinsic neurons. If the sympathetic and parasympathetic nerves to the gut are cut, many of the motor and secretory activities continue to occur because of control by the enteric nervous system.

The enteric nervous system is made up of interconnected neural plexuses and ganglia, which contain the nerve cell bodies. The myenteric and submucosal plexuses are the most well-defined plexuses in the wall of the GI-tract. The submucosal plexus lies in the submucosa, and the myenteric (Auerbach's) plexus is located between the longitudinal and

circular muscle layers. The majority of neurons controlling contractile activity in the small intestine have their cell bodies in the myenteric plexus. Axons of plexus neurons innervate smooth muscle cells in the muscularis externa and muscularis mucosa.

The majority of myenteric neurons are motor neurons to the longitudinal and circular muscle layers of muscularis externa. Excitatory motoneurons release acetylcholine (ACh) onto muscarinic receptors on smooth muscle cells; however these neurons also release tachykinins (TKs), which are direct excitants of muscle and also contribute to transmission (Furness et al., 1995). The TKs are a group of closely related peptides: substance P, neurokinin A, neuropeptide K, and neuropeptide Y (Mantyh et al., 1988). A majority of the excitatory motor neurons contain both ACh and TKs. Nevertheless, a minority of motor neurons may contain only ACh or only TKs (Furness et al., 1995). In addition to ACh and tachykinins, many excitatory muscle motor neurons contain opioid peptides (dynorphin, enkephalin, and opioid gene-related peptides). However, the opioid peptides do not seem to be primary transmitters, and generally have no direct effect on gastrointestinal muscle (Daniel et al., 1989).

Inhibitory motoneurons release nitric oxide (NO), adenosine triphosphate (ATP), peptide histidine isoleucine (PHI), and vasoactive intestinal peptide (VIP). Stimulation of the inhibitory neurons causes relaxation and

hyperpolarization of the smooth muscle cells (Smits and Lefebvre, 1996). The enteric inhibitory neurons do not have a single transmitter. It is possible that each of them contributes to inhibitory transmission to gastrointestinal muscle but to differing extents depending on region and species. Moreover, significant interplay occurs between the transmitters in that they can influence each other's release and action (Furness et al., 1995).

Functional experiments with rat ileum longitudinal muscle revealed only the involvement of NO (Kanada et al., 1992) and not VIP or ATP in inhibitory neurotransmission (Manzini et al., 1986). However, morphological studies also showed the presence of VIP immuno-reactive nerve cell bodies and fibers in the rat ileum (Schultzberg et al., 1980) and recently, coexistence of ATP and NO was reported in myenteric neurons of the rat ileum (Belai and Burnstock, 1994). Investigations of transmission in the rat intestine indicated that ATP could contribute significantly to transmission in the duodenum but not in the ileum (Manzini et al., 1986). The enteric nervous system interfaces directly with the intestinal smooth muscle and provides moment-to-moment control of contractile activity.

1.5.3 Chemical Control

Chemical control involves the stimulation or inhibition of smooth muscle contractile activity by humoral substances

that may act through neurocrine, paracrine or endocrine modes. Neurocrine substances are released from nerve endings or varicosities to affect adjacent smooth muscle. Paracrine substances are released from enteroendocrine or inflammatory cells within the gut wall while endocrine effects occur from blood-borne substances whose site of action is remote from the point of release. Examples of these regulatory substances include histamine, serotonin, opioids, cholecystokinin, motilin, somatostatin, vasoactive intestinal peptide and substance P. These substances can modulate contractile activity in the small intestine. The chemical stimulation controls the amplitude, duration and frequency of and the work performed by each contraction, as well as the direction, distance and velocity of propagation (Otterson and Sarr, 1993).

There is also an intimate relation between the immune system of the gut and the enteric nervous system that may extend to the motor activity of the small intestine. The best-recognized immune mediator is histamine, which is found within the mast cells of the gut. When mast cells degranulate, the enteric nervous system is activated, and specific patterns of contractile activity may be initiated. An example of the interaction of immune mechanisms may be Crohn's disease. The ganglion cell content of enteric nervous system of patients with Crohn's disease is increased, and the myenteric plexus becomes stimulated by plasma cells, mast

cells, macrophages and lymphocytes (Otterson and Sarr, 1993).

1.6 Consequences of Altered Motor Functions

Disturbances of motor function produce a variety of symptoms, including dysphagia in the esophagus, bloating, nausea and vomiting in the stomach and proximal small intestine, and diarrhea or constipation in the remainder of gut. These symptoms can arise in the context of inflammation or immune activation in various gut regions and embrace such common entities as esophagitis, gastritis and idiopathic inflammatory bowel disease (IBD). Disruption of normal motor function leads to proliferation of enteric flora or the prolongation of enteric infections (Collins, 1996).

Changes in gastrointestinal motility can also affect the pharmacokinitics of orally administered drugs by altering the rate of delivery, bioavailability or mucosal absorption of the drug (Hebbard et al., 1995). In general, the rate of absorption and time taken to achieve maximal plasma concentration for well absorbed drugs may be modified by changes in gastrointestinal motility, but overall bioavailability is not usually affected (Hebbard et al., 1995). However, for poorly absorbed drugs, both the rate of absorption and bioavailability are likely to be altered. In patients with gastrointestinal motility disorders, drugs administered in a controlled release formulation, or those with poor bioavailability, are most likely to have a poorly

predictable therapeutic effect (Hebbard et al., 1995).

1.7 Oxygen Free Radicals and Intestine

The small and large intestine are particularly well endowed with the enzymatic machinery necessary for the production of large amounts of reactive oxygen metabolites (ROMs). Sources of radicals in the gastrointestinal tract include mucosal xanthine oxidase, amine oxidase and aldehyde oxidase as well as NADPH oxidase found in the resident phagocytic leukocytes (Parks et al., 1983). Oxidases found in the resident phagocytic cells, microvascular endothelium and mucosal epithelium appear to be the major sources of ROMs in the intestine.

The intestine is the richest source of the enzyme xanthine oxidase. Within the small intestine, the activity of xanthine oxidase is found primarily in the mucosal layer, with an increasing gradient of activity from villus base to tip, whereas in the colon the enzyme is equally distributed among the mucosa and the muscle (Van der Vliet et al., 1989). There is also a gradient of xanthine oxidase activity within the mucosa from duodenum (~ 10 U/g protein) to ileum (~ 3 U/g protein). The lamina propria, muscularis, nuclei, brush border and goblet cells contained negligible or no xanthine oxidase.

Most cells of the gastrointestinal tract possess numerous antioxidant enzymes and scavengers to protect

themselves from injurious radicals. Protection is offered by enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Freeman and Crapo, 1982). Literature data so far show conflicting results related to antioxidants in the GI-tract. Relatively high SOD levels were found in the mucosa of the stomach and the distal colon, while the SOD activity is lower in the mucosa from the small intestine (Van der Vliet et al., 1989). In contrast to this observation, Loven et al., (1982) demonstrated higher SOD activities in the mucosa from the Streptozotocin-diabetic rat small intestine compared with the large intestinal mucosa. A less marked gradient in SOD activity was seen in the muscular parts of the GI-tract.

No differences were observed in GSH amounts between the mucosa and muscle of the gastrointestinal tract (Van der Vliet et al., 1989). GSSG levels did not vary much within the GI-tract, and no large deviations are found in the GSH/GSSG ratio, which suggests that the oxidant/antioxidant status was approximately constant throughout the GI-tract. From the duodenum to the distal colon, the activity of GSH-px increased slightly (Van der Vliet et al., 1989). The longitudinal gradient for catalase is approximately the same as that for GSH-Px. However, higher catalase activity was demonstrated in the muscle of small intestine compared with the colon (Van der Vliet et al., 1989).

Furthermore, the mucosa from the intestine is covered

with a substantial amount of mucus, a mixture of glycoprotein polypeptides which have antioxidant activity (Cross et al., 1984) and may provide protection to the surface epithelium of the gastrointestinal tract by scavenging oxidants such as H_2O_2 and ·OH within the lumen. The very high concentration of sugars in mucus secretions (approximately 40 mg/ml in adherent gastric mucus) should give them a substantial capacity to scavenge ·OH, and protein core of the glycoproteins in the mucus is also attacked and split by ·OH radicals (Cross et al., 1984). Balasubramanian et al., (1988) also showed that the resistance of intestinal mucosa to lipid peroxidation is due to the presence of a novel inhibitor which is lipidic in nature. The intestinal epithelium could also plays a role in the protection against H_2O_2-induced damage, possibly by its metabolizing capacity (Van der Vliet et al., 1992).

Generally, the colon has higher levels of GSH, GSH-Px and SOD compared with the small intestine (Van der Vliet et al., 1989), and within the small intestine SOD, GSH-Px and catalase levels are higher in the muscle than the mucosa. Taken together, enzyme measurements suggest that the small intestine is more vulnerable to oxygen radical damage than the colon.

The rate of production of reactive oxygen metabolites may exceed the capacity of the antioxidant defenses, thus resulting in tissue damage. Reactive oxygen metabolites play

an important role in mediating cellular injury associated with gastrointestinal diseases. The involvement of oxygen radicals have been demonstrated following active episodes of small intestinal ischemia/reperfusion (Parks et al., 1982), and inflammation (McCord and Roy, 1982).

1.7.1 Role of Oxygen Free Radicals in Ischemia-Reperfusion

Post-ischemic intestinal tissue damage is induced, at least partly, by the enhanced formation of oxygen radicals (Granger et al., 1986; Schoenberg and Beger, 1996). Oxidant formation has been demonstrated during reperfusion of the ischemic bowel by electron-spin resonance spectrometry and chemiluminescence (Zimmerman and Granger, 1994). The initial source of oxygen radicals is the hypoxanthine-xanthine oxidase system. The assumption that xanthine oxidase is the primary source of oxygen radicals in ischemic tissues is based on the observation that xanthine oxidase inhibitors reduced intestinal ischemia/reperfusion-induced injury (Gross et al., 1994; Granger et al., 1986).

During the ischemic period, ATP is catabolized to hypoxanthine, which accumulates in the tissues. Normally, the hypoxanthine level in the intestine ranges between 20-40 μM but it can increase to greater than 400 μM during ischemia (Granger et al., 1986). As a result of the low energy state in ischemia, there is an influx of Ca^{2+} into the intestinal cells. The intracellular Ca^{2+} then triggers the conversion of

NAD^+-reducing xanthine dehydrogenase to the oxygen radical-producing xanthine oxidase via proteolysis. When the intestine is reperfused, molecular oxygen is reintroduced into the tissues and reacts with hypoxanthine and xanthine oxidase to produce a burst of superoxide anion and hydrogen peroxide (Granger et al., 1986).

Although the studies implicate the superoxide anion in mediating reperfusion-induced changes in intestinal integrity, there is evidence suggesting that oxidants derived from superoxide play a more important role in the injury process. In as much as the intestine is a rich source of iron, it is quite possible that the iron-catalyzed Haber-Weiss reaction is involved in the production of $\cdot OH$ after reperfusion. Although iron is normally stored in enterocytes in the form of ferritin micelles, superoxide can react with the Fe^{3+} in ferritin to liberate Fe^{2+}. Thus, xanthine oxidase-generated superoxide could provide ferrous ions for reaction with H_2O_2 to form $\cdot OH$ in the post-ischemic intestine.

The assumption that the hydroxyl radical plays a critical role in ischemia/reperfusion injury is supported by data showing the protective effect of dimethyl sulfoxide (DMSO), a hydroxyl radical scavenger (Gross et al., 1994), and other non-enzymatic scavengers of hydroxyl radical, including dimethylthiourea and mannitol (Gross et al., 1994; Zimmerman and Granger, 1994). The role of iron in reperfusion-induced hydroxyl radical production has been

established from the observation that deferoxamine (an iron chelator) or apotransferrin (an iron-binding protein) provide protection against ischemia reperfusion (I/R)-induced increase in permeability of small intestine (Zimmerman and Granger, 1994).

1.7.2 Role of Oxygen Free Radicals in Inflammatory Bowel Diseases

The term inflammatory bowel diseases (IBD) describes two intestinal disorders; ulcerative colitis, a disease that occurs in the colon and is accompanied by rectal bleeding, diarrhea and pain, and Crohn's disease, which can affect most of the gastrointestinal tract and is accompanied by abdominal pain, diarrhea, vomiting, fever, infection and weight loss.

Increased numbers of granulocytes, including neutrophils, are found in the gut wall of patients with IBD. Neutrophils can generate reactive oxygen metabolites (ROMs) such as H_2O_2 and certain N-chloramines (NH_2Cl). These ROMs are thought to be important mediators of cellular damage (Hyslop et al., 1988). It has been suggested that superoxide anion or hydroxyl radical may be the major oxygen radicals contributing to the injury to small intestine (Wengrower et al., 1987; Gross et al., 1994); however, H_2O_2, on the other hand, has also been detected in inflammatory disease. The demonstration that $\cdot OH$ is generated *in vitro* by polymorphonuclear leukocytes, monocytes and macrophages

(Babior et al., 1975) and that it may have antibacterial properties suggests that it may be generated at sites of inflammation.

There is a significant correlation between the activity of free radicals in the intestine and the severity of inflammatory bowel disease (Wengrower et al., 1987). Seims et al., (1992) demonstrated in colitis an accumulation of free radical-mediated components of tissue damage. Furthermore, oxygen radicals generated by local infusion of hypoxanthine-xanthine oxidase into cat mesenteric artery are capable of increasing vascular permeability (Gross et al., 1994). Ahnefelt-Ronne et al., (1990) found increased lipid peroxidation in rectal biopsies of inflammatory bowel disease patients-another indication of oxygen radical-mediated tissue injury.

Various free radical scavengers have usually, but not always, shown beneficial effects in different animal models of intestinal inflammation. Detoxification of superoxide and hydrogen peroxide with SOD and catalase significantly attenuated the chemically-induced colitis (Gross et al., 1994). There have also been a few preliminary clinical reports dealing with the possible beneficial effects of free radical scavengers in inflammatory bowel disease. Emerit et al., (1989) reported that the intra-muscular administration of SOD in patients with Crohn's disease significantly improved the clinical situation. Levin and coworkers, (1992)

demonstrated beneficial effects of allopurinol, which is a xanthine oxidase inhibitor. A larger double-blind, randomized, endoscopically clinical trial was carried out by Salim, (1992). He concluded that sulfasalazine and 5-aminosalicylic acid, two important drugs used in the treatment of inflammatory bowel disease, are reactive oxygen scavengers, furthering the idea that oxygen radicals play a fundamental role in gastrointestinal inflammation.

1.7.3 Oxygen Free Radicals and Altered Motility of Intestine

The occurrence of oxygen free radicals in the circulation has been established. These may be locally generated, (i.e. IBD and ischemia-reperfusion) or exogenously delivered by the xanthine oxidase reaction.

The motility apparatus of the gut includes smooth muscle, extrinsic and intrinsic nerves and endocrine cells. An inflammatory process in the gut can alter motility by killing these cells or by influencing their function. Factors produced by cells of an inflammatory infiltrate can induce structural changes in these cell types, influence their growth or modulate their function through potentiating or inhibitory interactions at various steps in the excitation-response sequence. Inflammation restricted to the mucosa can affect the function of the muscularis externa, and there is a relationship between altered motility and the presence of inflammation in patients with IBD (Kern et al., 1951).

Inflammation-induced changes in muscle contraction are region-specific, are different between circular and longitudinal muscles and appear independent of the type of injury.

There are few studies of small intestinal motility in patients with IBD. One study demonstrated abnormal motor activity in the proximal small intestine of patients with ulcerative colitis (Collins, 1996). There is also increased contractility in muscle resected from the small intestine of patients with Crohn's disease (Vermillion et al., 1993); however, a reduction in contractility was observed in muscle from patients with colitis (Snape et al., 1991).

There is also evidence that inflammation at one site in the gut may altered motor function at a remote non-inflamed site, and motor abnormalities have been shown in the stomach and small bowel of patients with ulcerative colitis. These findings suggest that inflammation can also induce changes in enteric nerves at remote non-inflamed sites as shown by Dvorak et al., (1979).

Hypoxia-reoxygenation significantly alters intestinal motility. The generation of reactive oxygen species and disruption in the calcium homestasis play an important role in the pathogenesis of reoxygenation damage. During hypoxia, the frequency of the spontaneous contractions and the resting tension decreased (Bielefeldt and Conklin, 1997). Also, ischemia caused significant decrease in muscle contractile

force, oxygenation and superoxide dismutase activity, and reperfusion of ischemic muscle increased the muscle contractile force (Tuncel et al., 1997).

Direct studies involving the exogenous administration of OFRs *in vivo* and *in vitro* and corresponding changes in intestinal motility are currently lacking. There are only a few studies have been done by Matthews and Mesler, (1984); Van der Vliet et al., (1989); and Moummi et al., (1991b). Photon activation of the halogenated fluorescien derivative erythrosine through the generation of singlet oxygen caused marked calcium-dependent contraction of the smooth muscle cells of the guinea pig taenia coli superfused *in vitro* (Matthews, and Mesler, 1984). Van der Vliet et al., (1989) studied the effects of H_2O_2 and cumene hydroperoxide on the longitudinal muscle function. A short-lasting contraction followed by a relaxation was observed. Also Moummi and coworkers, (1991b) reported that several oxygen metabolites (H_2O_2, HOCl and NH_2Cl) caused contraction of guinea pig ileum smooth muscle, via the release of prostaglandins and possibly neurotransmitters from intramural nerves.

It is quite evident from the above studies that the role played by OFRs in the modulation of intestinal smooth muscle tone is unclear. In addition, the literature also suggests that it is unclear as to which OFR is producing contraction or relaxation of smooth muscle. There is no study on the effect of $\cdot OH$ and $O_2^{\cdot -}$ on intestinal smooth muscle cells.

However, based on conclusion on the well known fact that ·OH can be generated by the interaction of $O_2^{\cdot -}$ and H_2O_2, it is suggested that ·OH may also play a role in the smooth muscle tone.

1.7.4 Mechanisms Underlying Altered Motility: Mediators Responsible for Altered Muscle Function

1.7.4.1 Prostaglandins

The prostaglandins (PGs) are a family of hydroxy fatty acids derived from 20-carbon polyunsaturated precursors found in the cell membrane of all mammalian tissues. The predominant PG precursor is arachidonic acid (AA), released from membrane phospholipids by phospholipase A_2. Arachidonic acid can be enzymatically metabolised to two main groups of compounds. Enzymatic degradation by cyclooxygenase leads to the formation of PGH_2, which is subsequently converted into a range of prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) and thromboxane A_2 (TXA_2). Leukotrienes (LTs) are formed during processing of arachidonic acid in the lipoxygenase pathway. The initial step is the synthesis of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), from which 5-hydroxyeicosatetraeonic acid (5-HETE) and LTA_4 are derived. Further enzymatic processing of LTA_4 gives rise to the LTB_4, LTC_4, LTD_4 and LTE_4 (McKay and Perdue, 1993).

Normally the subepithelial component of the intestine produces most of the PGs, although epithelial cells are

certainly capable of producing eicosanoids (McKay and Perdue, 1993; Eberhart and Dubois, 1995). The cells most likely to be responsible for eicosanoid production include immune cells in the lamina propria and subepithelial mesenchymal cells, in particular, mast cells and phagocytes, are known to be avid producers of eicosanoids. PGE_2 is the major prostanoid in the subepithelium, whereas $PGF_{2\alpha}$ predominates in the epithelium (Lawson and Powell, 1987; Eberhart and Dubois, 1995). It is estimated that >70% of colonic PGE_2 is metabolized in the epithelium (McKay and Perdue, 1993).

There are several lines of evidence which indicate that endogenous PGs may play a role in regulating the motility of gastrointestinal smooth muscle. Firstly, PGs are produced and released from the mucosal layer and muscle of the gastrointestinal tract. Secondly, prostaglandins are released during muscle activity, and thirdly changes in the motility of the intestine induced by inhibitors of PG synthesis correlate with changes in the concentration of endogenous PGs (Kubota et al., 1982).

The action of PGs on GI muscle tone is extremely variable, depending on the type of PG, the concentration, the organ, species, and even the muscle layer studied. In general, prostaglandin I (PGI_2) relaxes smooth muscle preparations (Lawrence et al., 1992) and several studies have shown that PGI_2 inhibits changes in motility induced by other PGs (Bennett and Fleshler, 1970; Eberhart and Dubois, 1995).

However, PGI_2 at high concentrations contracts the longitudinal muscle of the guinea pig ileum due to stimulation of prostaglandin I (IP) receptors present on the smooth muscle cells (Lawrence et al., 1992). This contraction was abolished by atropine and shows that PGI_2-induced contractions are mediated by ACh release from cholinergic neurons as a consequence of increased excitability of the cell bodies (Gaion and Trento, 1983). The presence of IP receptors on enteric neurons and/or sensory nerve terminals has been confirmed (Lawrence et al., 1992).

The investigation of small intestinal motility in most species shows opposing effects of PGE_2 and $PGF_{2\alpha}$, both of which are produced in the small intestine. Isolated small intestine longitudinal and circular muscle layers respond to PGE_2 and $PGF_{2\alpha}$ in the same manner as the colon. *In vivo*, PGE_2 caused inhibition of myoelectric and mechanical activity at low doses (Tollstrum et al., 1988) and marked excitation at higher doses (Burakoff and Percy, 1992), whereas $PGF_2\alpha$ increases electrical activity of the migrating motor complexes (Tollstrum et al., 1988). However, *in vitro*, PGE_2 caused excitation of both the proximal and distal colon (Burakoff and Percy, 1992). Both PGE_1 and PGE_2 contract the intestinal longitudinal muscle and relax the circular muscle, while $PGF_{2\alpha}$ contracts both types of smooth muscle cells *in vitro* (Bennett et al., 1968; Bennett and Fleshler, 1970).

The excitatory actions of the PGs seem due to direct

action on smooth muscle cells. However, part of their action is mediated by cholinergic and non-cholinergic intramural nerves (Bennett et al., 1975). PGE_2 influences intestinal motility by acting directly or at a neuronal level (Botella et al., 1993). The stimulatory effect of $PGF_{2\alpha}$ is also partly dependent on calcium and probably not dependent on the autonomic or the enteric nervous system (Eberhart and Dubois, 1995). However, the inhibitory effects of PGE_2 were seen to be partially mediated by increased activity of sympathetic nerves (Kadlec et al., 1974).

Prostaglandins act through specific receptors which have been subdivided into PGE receptors, subtypes EP_1, EP_2, and EP_3, on the basis of the relative potency of selective agonists and antagonists in both functional and binding studies; prostaglandin F receptor (FP), and prostacyclin receptors (IP) (Botella et al., 1993; Narumiya, 1994). *In vitro* experiments have shown that EP_1 and EP_3 receptors mediate contraction of the ileum longitudinal smooth muscle, whereas EP_2 receptors mediate relaxation of ileum (Botella et al., 1993).

Whether or not inflammation due to oxygen radicals is mediated by prostaglandins is being debated. Prostanoids may be involved in inflammatory disease of the intestine. Increased mucosal PG synthesis has been reported in inflammatory bowel disease, and these compounds have been implicated in the pathogenesis of diarrhea in these

conditions (Burakoff and Percy, 1992). Kao and Zipser, (1988) demonstrated that colitis was associated with a greater than five-fold increase in PGE_2. Prostaglandins have also been implicated in the pathogenesis of intestinal ischemia. These substances are released from ischemic small bowel (Granger et al., 1986).

The link between free radicals and PGs is supported by several studies. Hydrogen peroxide stimulated PGE_2 and/or PGI_2 production in mast cells, fibroblasts and endothelial cells (Lewis et al., 1988). The mechanism of stimulation is unclear, but in the endothelial cells it seems to involve gating of Ca^{2+} across the cell membrane either as the result of a receptor-mediated event or secondary to lipid peroxidation of the cell membrane, making it more permeable to Ca^{2+} with subsequent liberation of arachidonic acid from membrane stores (Lewis et al., 1988).

Reactive oxygen metabolites are also able to stimulate phospholipase A_2 and 5-lipoxygenase directly, and thus may promote formation of leukotrienes and prostaglandins (Otamiri et al., 1988). Oxidants generated by xanthine /xanthine oxidase increased PGE_2 (~5-fold) ,and 6-keto $PGF_{1\alpha}$ (~15-fold) tissue levels (Bern et al., 1989).

Several potential mechanisms have also been suggested whereby H_2O_2 might stimulate production of prostaglandins in the intestine. Peroxides are important cofactors ("peroxide tone") for prostaglandin biosynthesis, as demonstrated by

experiments involving the exogenous addition of either H_2O_2 or lipid peroxides to tissues (Weiss et al., 1979; Bern et al., 1989). However, there may be an important dose-response effect in that high levels of lipoperoxides, perhaps through ·OH formation, will inactivate prostaglandin and prostacyclin synthases (Weiss et al., 1979), and low concentrations of hydroperoxides have ability to activate PGH synthase and stimulate prostaglandin production (Marshall and Lands, 1986). Another possible mechanism for peroxide stimulation of prostaglandin production is the activation of inflammatory or mesenchymal cells (Bern et al., 1989).

1.7.4.2 Histamine

Histamine is produced by decarboxylation of histidine. L-histidine can be decarboxylated by both histidine decarboxylase (HDC) and L-dopa decarboxylase. In rat intestine, the highest level of enzyme activity occurs in the cecum, with intermediate levels in the small intestine, and the lowest in the colon (Rangachari, 1992).

Histamine is produced by enterochromaffin cells (ECL) as well as mast cells. There are at least two types of mast cells in the intestine. The mucosal mast cells are found primarily in the mucosa, whereas the connective tissue mast cells are found in the submucosa and muscularis (Kanwar and Kubes, 1994). A variety of mediators are released through mast cell activation and degranulation, such as histamine,

serotonin, platelet activating factor and eicosanoids which are known to affect the motor and secretory activity of the digestive tract (Rangachari, 1992).

Stored histamine can be released after degranulation of the cell by two general processes: cytolytic and non-cytolytic release. In the first instance, in which the plasma membrane is damaged, the process is energy independent, does not require intracellular Ca^{2+}, and is accompanied by leakage of cytoplasmic constituents. Non-cytolytic release can occur by both antibody-dependent and antibody-independent mechanisms (Rangachari, 1992). Antibody-dependent mechanisms involve cross-linking of immunoglobulin E receptors on mast cells that initiates a series of biochemical events leading to the liberation of stored mediators. Several peptides have been shown to release histamine by an antibody-independent mechanism. These include neuropeptides, such as substance P and kinins, complement fragments, and macrophage-derived factors (Church et al., 1989).

Histamine has both secretory and contractile effects on the small and large intestine in a number of species. There are also regional variations in sensitivity to histamine, with ileal and jejunal strips being more sensitive than duodenal or colonic strips (Rangachari, 1992). Histamine receptors are found in ganglia of the myenteric plexus, on intestinal smooth muscle of most mammals, as well as on cells of the immune system such as human basophils and the rat

lymphocyte. Longitudinal muscles appear to have only H_1 receptors, whereas circular muscles have both H_1 and H_2 receptors (Yamanaka and Kitamura, 1987).

Histamine may act directly on smooth muscle to yield a species-and region-dependent response (contraction, relaxation), or no response, or indirectly through neurons of the plexus to alter smooth muscle activity. The direct contractile effect is mediated through occupation of histamine H_1 receptors. Histamine receptor (H_2) stimulation appear to relax intestinal strips, but these effects are seen at high concentrations (Rangachari, 1992). The indirect effect of histamine may be due to histamine-evoked release of acetylcholine, substances P, 5-hydroxytriptamine (5-HT), or PGs from the myenteric plexus (Barker and Ebersole, 1982).

In the intestinal ischemia/reperfusion model, some experiments support the view that histamine is released during ischemia. Histamine release from the ischemic intestine is due to the action of oxygen free radicals generated during reperfusion (Boros et al., 1991). The increased flux of oxidants previously described at the onset of reperfusion may be responsible for mast cell activation (Kanwar and Kubes, 1994). This is consistent with the observation of Boros et al., (1991) that antioxidants blocked histamine release from the postischemic gut. The finding that allopurinol, xanthine oxidase inhibitor blocked by 87% histamine release from post ischemic gut also suggests an

important role for the oxidant-generating enzyme xanthine oxidase-induced mast cell activation (Boros et al., 1989).

Mast cells have also been implicated in the pathophysiology of IBD. In IBD, there are increased numbers of mast cells in the muscle layers and connective tissue and there is evidence of altered function of intestinal mast cells isolated from patients with IBD. Intestinal mast cells may contribute to motility disturbances in the inflamed gut by responding to stimuli arising from altered neurotransmitter release, specific antibodies, or other immunological factors produced as part of the inflammatory reaction (Fox et al., 1993).

The link between the generation of free radicals and a histamine-mediated inflammatory reaction was initially provided by Ohmori et al., (1978) showing that injecting a mixture of hypoxanthine and xanthine oxidase (HPX-XOD) produced foot edema, most effectively suppressed by the antihistamine diphenhydramine. More direct evidence was provided by this author that HPX-XOD released histamine from isolated rat mast cells without causing nonspecific lysis of the cells. Hydrogen peroxide was thought to be involved in the releasing reaction. Moreover, incubation of rat peritoneal mast cells with hydrogen peroxide resulted in a significant release of histamine without any concomitant release of lactate dehydrogenase (LDH) (Ohmori et al., 1980; Mannaioni and Masini, 1988).

Release of mast cell histamine could be accounted for by a direct interaction of oxy-radicals with mast cell membranes (Mannaioni and Masini, 1988). Hydrogen peroxide can interact with catalytically active iron and generate highly reactive oxidants, which in turn are responsible for histamine release (Boros et al., 1991).

A link between the release of arachidonic acid, its metabolic activation into a free radical and the release of mast cell histamine has also been provided by some experiments tentatively coupling two basic steps in inflammation, such as prostaglandin and leukotriene production and histamine release (Masini et al., 1987). However, the release of mast cell histamine by activated arachidonic acid has a long time-course, and is not accompanied by a significant leakage of LDH (Masini et al., 1987).

1.7.4.3 Acetylcholine

Acetylcholine (ACh) is a primary transmitter of excitatory enteric neurons that innervate gastrointestinal muscles (Furness et al., 1988). Postganglionic cholinergic neurons also innervate the smooth muscle of the gut, and ACh and other muscarinic agonists can cause contraction of gut smooth muscle (Berne and Levy, 1993).

Enteric neurons may be affected directly or indirectly, releasing neurotransmitters, including ACh. Neural damage has

been observed in biopsies of patients with Crohn's disease and ulcerative colitis (Brewer et al., 1990; Kubota et al., 1992). There are also abnormalities of structure and neurotransmitter content in the enteric plexuses of patients with IBD. Changes in the number of ganglia have been reported, together with evidence of axonal degeneration and infiltration by a variety of cells, including lymphocytes, mast cells, eosinophils and macrophages (Geboes et al., 1991). Studies involving quantification of radio-labeled neurotransmitter released from myenteric plexus preparations have shown substantial changes in the evoked release of both noradrenaline or acetylcholine during inflammation (Jacobson et al., 1993).

The link between the oxygen free radicals and ACh is supported by some studies. There is a strong possibility that ACh is released from cholinergic neurons by H_2O_2 (Tamai et al., 1991), and antioxidants protect neuronal cells *in vitro* from toxicity with oxidants (Miyamoto et al., 1989). However, Gaginella et al., (1992) have shown that endogenous prostaglandins, possibly liberated as a consequence of the oxidant injury, may be involved in the ACh release process. Another reactive metabolite, NH_2Cl at concentrations believed to exist in inflamed tissue, also causes the release of ACh from mucosal/submucosal nerves primarily through nonspecific neural membrane injury (Gaginella, et al., 1992; Tamai et al., 1991) and endogenous prostaglandins may be involved in

this ACh release process (Gaginella, et al., 1992). There is also an apparent relationship between oxidant-induced generation of prostaglandins and ACh-mediated secretion by the rat colon (Bern et al., 1989). Indeed, in a longitudinal muscle preparation of the guinea-pig ileum, prostacyclin and PGE_1 caused a contraction mediated by the release of acetylcholine from the myenteric plexus (Yagasaki et al., 1981); and atropine reduced the contraction of guinea pig ileum caused by PGE_1 (Bennett et al., 1968).

The reactive oxygen metabolites such as hydrogen peroxide may cause damage to the cell membrane and alter the coupling of the muscarinic receptor to its effector system (Moummi et al., 1991b). The contraction induced by muscarinic receptor stimulation has been shown to reduce by pretreatment of tissue with hydrogen peroxide, implying that the muscarinic receptor response may be affected (Van der Vliet et al., 1989). Isometric tension induced by muscarinic stimulation was less in muscle from rabbits with colitis compared to controls (Cohen et al., 1986). Other radicals such as NH_2Cl have also been shown to decrease significantly the sensitivity of the smooth muscle to carbachol, a muscarinic receptor stimulant (Moummi et al., 1991b).

1.7.4.4 ATP-Sensitive Potassium Channels

The motor activity of gastrointestinal smooth muscle is closely related to the membrane potential. Controlling the

membrane potential via modulation of potassium channels is essential for the action of neurotransmitters on smooth muscle. ATP-sensitive potassium channels (K^+-ATP channels) have been identified in cardiac muscle, skeletal muscle and smooth muscle (Edwards and Weston, 1993; Frank et al., 1994). The presence of glibenclamide-sensitive K^+ channels in rat ileal smooth muscle has been shown by Frank et al., (1994); however, the physiological and pathophysiological role of the these channels in intestinal smooth muscle was unclear. These channels do not seem to be involved in the effect of inhibitory mediators such as NO, ATP or calcitonin gene-related peptide (Frank et al., 1994). Opening of these channels causes hyperpolarization of the smooth muscle (Edwards and Weston, 1993).

ATP-sensitive potassium channels have been shown to be involved in ischemic responses in cardiac tissue (Benndorf et al., 1992). These channels have also been associated with vascular function in the intestine (Silberberg and Van Breeman, 1992), and in hemodynamic changes and a reduction of portal pressure in an experimental model of portal hypertension in the rat (Moreau et al., 1992).

The link between OFRs and K^+-ATP channels is supported by some studies. Oxygen free radicals generated by the reaction between purines and xanthine oxidase decreased the resting membrane potential, action potential amplitude and maximum rate of rise of action potential in guinea pig

ventricle (Pallandi et al., 1987). Goldhaber et al., (1989) also reported that the OFRs generated by xanthine and xanthine oxidase or H_2O_2 activate K^+-ATP channels. A decrease of the calcium current and an increase of the time-independent outward current perhaps due to opening of ATP-sensitive K^+ channels could be the possible mechanisms for the shortening of the action potential duration induced by OFRs (Goldhaber et al., 1989).

Direct evidence for opening of K^+-ATP channels was obtained from single channel current recordings in which OFRs increased the channel activity, and glibenclamide effectively prevented the opening of the channels (Tokube et al., 1996). The opening of K^+-ATP channels by OFRs has been attributed to an irreversible inhibition of both oxidative phosphorylation and glycolysis (Goldhaber et al., 1989) or by modulation of ATP binding sites on the K^+-ATP channels, without affecting ADP binding or glibenclamide binding sites (Tokube et al., 1996).

1.7.4.5 Nitric Oxide

Nitric oxide (NO) is a readily diffusible, highly reactive, labile substance. It is an oxy-radical because it has an unpaired electron associated with the oxygen atom. Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) which converts molecular oxygen and L-arginine to NO and L-citrulline. Nitric oxide synthase has been

detected in various cells and is classified into two main categories. Firstly, the constitutive NOS, present in nerve terminals, endothelial cells, intestinal epithelial cells, myenteric neurons and smooth muscle, which releases small amounts of NO (pmol) for short periods of time upon activation. Secondly, there is the inducible NOS, found in macrophages and vascular smooth muscle cells and intestinal epithelial cells, which releases larger amounts of NO (nmol) for long periods of time (Moncada, 1992; Bredet et al., 1990; Dignass et al., 1995).

Nitric oxide has well-described effects on motility and is believed to be the major inhibitory neurotransmitter in the gut. In many isolated gastrointestinal preparations, NO has been identified as the mediator partly or entirely responsible for non-adrenergic, non-cholinergic (NANC) relaxation (Kanada et al., 1992; Bartho and Lefebvre, 1994).

Nitric oxide could act directly on the muscle or could evoke the release of another substance (Furness et al., 1995). It has been shown to induce contraction at low concentrations (10^{-10} to 10^{-7} M) and relaxation at high concentrations in rat ileum (Bartho and Lefebvre, 1994; Smits and Lefebvre, 1996). Nitric oxide induce relaxation of both longitudinal and circular muscle of rat ileum (Kanada et al., 1992).

The signal transduction system for the smooth muscle relaxing actions of NO involves activation of soluble

guanylate cyclase and the production of second messenger cyclic guanosine monophosphate (cGMP), thereby inducing intestinal smooth muscle relaxation or modifying neurotransmission (Kanada et al., 1992; Rand and Li, 1995). The intracellular levels of cGMP are regulated by guanylate cyclase which catalyzes its formation from GTP (Mittal and Murad, 1982). Guanylate cyclase activity is distributed in the particulate and cytosolic fractions of most tissues (Mittal and Murad 1982).

The toxicity of NO is generally observed in the presence of reactive oxygen intermediates under conditions such as ischemia/reperfusion injury. Some studies suggest that tissue injury during ischemia/reperfusion is attributed to endogenously generated NO, and overstimulation of NO synthases might therefore contribute to these pathophysiological states. Increased NOS activity has also been observed in patients with ulcerative colitis and Crohn's disease (Boughton-Smith et al., 1993). Enhanced colonic NO generation by stimulated nitric oxide synthase activity in IBD may contribute to tissue injury. Indeed, in patients with ulcerative colitis, NO synthase activity was increased 8-fold in the mucosa (Boughton-Smith et al., 1993). Also, studies in colitis in the rat suggest that NO plays a selective role in the inflammatory process because NO inhibitors improved some but not all aspects of the colitis (Hogaboam et al., 1995).

As compared to the highly reactive hydroxyl radical, NO

free radicals are several orders less reactive. These radicals cause little or no increase in lipid peroxidation, the cardinal sign of oxidative stress. However, the reaction of NO with superoxide produces a labile non-radical compound, the peroxynitrite anion, which will spontaneously decompose by homolytic scission to yield powerful oxidants such as hydroxyl radical with a half life of less than 1 second (Chiueh et al., 1994).

The link between free radicals and NO production could be supported by the fact that the oxygen radicals/NO oxidatively activate guanylate cyclase (Mittal, 1995). The evidence is presented that oxygen radicals are required for the synthesis of nitric oxide by NO synthase as demonstrated by inhibition of NO formation by oxygen radical scavengers. Oxygen radicals and hydrogen peroxide participate in the catalytic conversion of L-arginine to nitric oxide by nitric oxide synthase in the presence of calcium ion (Mittal, 1995). The exact mechanism(s) whereby oxygen radicals participate in NO formation is not known, but Mittal, (1995) proposed that hydrogen peroxide could peroxidatively oxidize the guanidino nitrogen of L-arginine to NO by NO synthase.

Guanylate cyclase might also be regulated by free radicals. Both superoxide anion and H_2O_2 are required for activation of guanylate cyclase. These compounds can react by the Haber-Weiss or Fenton reaction to form hydroxyl radicals, a postulator activator of guanylate cyclase *in vivo*

(Waldman and Murad, 1987). Also, arachidonic metabolism and prostaglandin formation can increase levels of hydroxyl radical, and these processes are associated with elevated levels of cGMP as well as increases in guanylate cyclase activity (Waldman and Murad, 1987). Thus, it is possible that regulation of guanylate cyclase activity and cGMP formation may occur via mechanisms involving oxidative processes generating reactive free radicals that interact directly with guanylate cyclase.

1.7.5 Spontaneous Activity of the Ileum

In the absence of electrical stimulation, intact ileum preparations exhibit spontaneous contractions (Hong et al., 1997). Spontaneous activity mainly involves the longitudinal muscle-myenteric plexus (Hong et al., 1997). The main source of calcium for smooth muscle contractile responses derives from extracellular Ca^{2+} through voltage-gated L-type Ca^{2+} channels (Ozaki et al., 1991) and/or release of Ca^{2+} from internal stores by internal messengers such as phosphoinositides or by a Ca^{2+}-induced Ca^{2+} release mechanism (Toescue et al., 1995).

Spontaneous activity is related primarily to opening of L-type Ca^{2+} channels on the longitudinal muscle (Hong et al., 1997). The low resting membrane potential of longitudinal muscle reinforces the probability that L-type Ca^{2+} channels open spontaneously (Hong et al., 1997). When a large amount

of Ca^{2+} is transported into the myoplasmic compartment, the operation of large-conductance K_{ca} channels is enhanced to result in prolonged and generalized membrane hyperpolarization, a decrease in Ca^{2+} level and suppression of spontaneous activity (Hong et al., 1997).

Prostaglandins have also been shown to have distinct actions on the threshold potential at which an action potential is generated (Kubota et al., 1982). All prostaglandins suppress both the spontaneous spike discharges and the mechanical activity of the smooth muscle cell, and raise the threshold for the generation of action potentials (Kubota et al., 1982). In electrically and mechanically quiescent muscle cells, indomethacin reduced the threshold for action potential (Kubota et al., 1982). Prostacyclin inhibits the spontaneous activity of rat isolated colon (Qian and Jones, 1995) and prostacyclin receptor (IP receptor) activation is typically associated with inhibition of smooth muscle tone, including rhythmic activity of intestinal smooth muscle (Lawrence et al., 1992). Activation of IP receptors on enteric neurons in the rat colon causes a release of inhibitory neurotransmitters (Qian and Jones, 1995). Nitric oxide appears to be one of these transmitters; however, there is no support for ATP, adenosine or vasoactive intestinal peptide as the second transmitter candidate (Qian and Jones, 1995).

Spontaneous activity may be affected by OFRs. Hypoxia-

reoxygenation through the generation of reactive oxygen species and disruption in calcium homeostasis significantly alters intestinal motility. During hypoxia, the frequency of the spontaneous contractions and the resting tension decreased (Bielefeldt and Conklin, 1997). *In vitro* study by the Van der Vliet et al., (1989) also showed that H_2O_2 and cumene hydroperoxide diminished the spontaneous motility of the longitudinal smooth muscle, but they could not determine the mechanism of this process.

2.0 HYPOTHESIS

Considerable evidence suggests that several pathological situations in the gastrointestinal tract are characterized by the formation of oxygen free radicals, such as intestinal ischemia and inflammation of the intestine. One feature seen in these situations is disturbed intestinal motility. To date, studies on the pathophysiology of inflammatory bowel disease (IBD) have focused on the effects of free radicals on changes in the structure or function of cells in the mucosa and lamina propria. Little attention has been paid to the role of smooth muscle and enteric nerves in the pathophysiology of IBD. However, the long-standing observations that active IBD is accompanied by changes in motility, as well as by structural abnormalities in the enteric nervous system, strongly indicate that the structure and function of neuromuscular tissues in the gut are altered in IBD.

The intestinal mucosa is the most vulnerable region of the gastrointestinal wall in both ischemia and inflammation (Parks et al., 1983). However, changes in intestinal motility are described as a consequence of inflammation (Mayer et al., 1988), which implies that smooth muscle function of the intestine can also be disturbed by oxygen radicals. Also,

over the past few years there has been a great deal of new information about oxygen free radical-induced changes in smooth muscle contractility (e.g. in aorta and trachea). However, there have been no studies on oxygen free radical-induced changes in intestinal motility and no clear consensus as to the comparative role of oxygen metabolites in this motility.

Based on the above information, it is hypothesized that oxygen free radicals could produce changes in intestinal motility (contraction/relaxation). It is not known which of the oxygen free radicals are involved in contraction and/or relaxation of the small intestine. Also not known are their mechanisms of action. These free radicals can produce contraction and/or relaxation of small intestine. The contraction induced by these free radicals could be direct or mediated through the release of constrictor substances such as prostaglandins, histamine or acetylcholine. The relaxant effects could be direct or mediated through the release of nitric oxide and/or prostaglandins. It is, therefore, proposed to study comprehensively the effects of exogenously generated free radicals ($O_2^{\cdot -}$, $^{\cdot}OH$ and H_2O_2) on the isolated rat ileum, and to elucidate the mechanisms of oxygen free radical-induced contractile/relaxant responses of rat ileum.

3.0 OBJECTIVES

Main objectives were to investigate:

I) Which of the oxygen free radicals ($O_2^{\cdot -}$, $^{\cdot}OH$, H_2O_2) is/are involved in contraction/relaxation of rat ileum.

II) The mediators involved in OFR-induced contraction and/or relaxation of the rat ileum.

To achieve these objectives, the following protocol was developed:

Objective I

Objective I was achieved by using various oxygen free radicals and their scavengers (metabolizers); $O_2^{\cdot -}$ (superoxide dismutase), H_2O_2 (catalase), $^{\cdot}OH$ (mannitol, dimethylthiourea) and the $^1O_2$ (histidine). Tension measurements on isolated rat ileum were recorded for 20 minutes. Antioxidants were administered 3 min (superoxide dismutase and catalase) and 20 min (mannitol, dimethylthiourea, histidine) prior to addition of oxygen free radicals.

Objective II

To achieve objective II, the following studies were conducted on isolated rat ileum. Tension measurements were recorded for 20 minutes. Antagonists to contracting mediators (indomethacin, a prostaglandin synthesis inhibitor; atropine,

an antimuscarinic drug; pyrilamine, a histamine receptor antagonist) /or relaxing mediators (indomethacin; L-NMMA, an inhibitor of nitric oxide synthase; methylene blue, an inhibitor of guanylate cyclase; glibenclamide, an inhibitor of ATP-sensitive potassium channel) were administered 20 minutes prior to OFR administration.

Mechanisms of Contraction

To determine the mechanisms of OFR-induced contraction, the effects of OFR(s) were investigated :

i) in the presence of prostaglandin synthesis inhibitor (indomethacin).

ii) in the presence a of histamine receptor antagonist (pyrilamine).

iii) in the presence of an anti-muscarinic drug (atropine).

Mechanisms of Relaxation

To determine the mechanisms of OFR-induced relaxation, the effects of OFR(s) were investigated :

i) in the presence of an inhibitor of cyclooxygenase-derived metabolites (indomethacin).

ii) in the presence of a nitric oxide synthase inhibitor (N^G-monomethyl-L-arginine).

iii) in the presence of a guanylate cyclase inhibitor (methylene blue).

iv) in the presence of glibenclamide, a blocker of ATP-sensitive potassium channels.

4.0 MATERIALS AND METHODS

4.1 Preparation of Ileum Strips for Tension Measurement and Functional Experiments

Male Wistar rats (200-250 g) were anesthetized with a intra-peritoneal injection of pentobarbital sodium (60 mg/kg). The abdomen was opened, a length of ileum was removed and placed in a dish containing cold Krebs-Ringer solution of the following composition in meq/L : Na, 140 ; K, 4.6 ; Ca, 4.9 ; Mg, 2.3 ; HCO_3, 21.91 ; PO_4, 3.48 ; SO_4, 2.32 ; Cl, 125 ; and 5 mM glucose (pH 7.4). The mesentery is trimmed away and 1.5-2.0 cm strips were cut from the length of ileum. After rinsing the contents of strips, one end of the strip was tied to a stainless steel wire stirrup at the base of 10 ml organ bath filled with Krebs-Ringer solution, while the other end was attached to a Grass FT-03 force displacement transducer attached to a Grass polygraph recorder for recording responses (contraction or relaxation) of muscle strips. The solution in the bath was maintained at 37°C and constantly bubbled with a mixture of 95% O_2 and 5% CO_2. All preparations were allowed to equilibrate for 60 minutes at a resting tension of 1.0 g and were washed at 15 minute intervals. Acetylcholine (0.5 μg /ml), which produces a sub-maximal contraction of ileum, was used in all

experiments to assess the viability of each preparations. The response of the preparation to acetylcholine was recorded for 5 minutes and the preparation were washed at 5 minute intervals until the tension returned to the resting level.

In the functional experiments, agonists (free radicals) and their effects on the rat ileum were monitored for a total period of 20 minutes. After this treatment, the preparations were washed every 5 minutes until the tension returned to the resting level. Subsequently, acetylcholine was added to the organ bath and response was recorded for 5 minutes. All antioxidant or mediator antagonists were added 20 minutes prior to the addition of oxygen free radicals generating system with the exception of superoxide dismutase and catalase which were added 3 minutes prior to addition of OFRs.

Developed tensions were measured in mg/mg tissue and expressed as percentage of baseline tension.

4.2 Generation of Oxygen Free Radicals

4.2.1 Superoxide Anion Generation

Xanthine (X) and xanthine oxidase (XO) were used to generate superoxide anion ($O_2^{\cdot -}$)(Prasad et al., 1989). Superoxide anion is produced by the following reaction:

$$\text{Xanthine} + 2O_2 \xrightarrow[XO]{} 2O_2^{\cdot -} + 2H^+ + \text{uric acid} \qquad [4.1]$$

The three concentrations of X and XO used in these studies

were termed 1X, 2X, and 4X as follows:

(1X)-xanthine (10 μM) and xanthine oxidase (0.025 U/ml);

(2X)-xanthine (20 μM) and xanthine oxidase (0.05 U/ml);

(4X)-xanthine (40 μM) and xanthine oxideas (0.10 U/ml).

Solutions of xanthine were made in Krebs-Ringer solution and 0.1 to 0.4 ml of the solution was added in the organ bath to elicit responses. Xanthine oxidase was obtained from the Sigma Chemical Company as a suspension from milk (0.1 U/mg protein) which was added directly into the bath containing the ileum segments.

4.2.2 Hydroxyl Radical Generation

Various concentrations of dihydroxy fumaric acid (DHF), ferric chloride ($FeCl_3$) and adenosine diphosphate (ADP) were used to generate the hydroxyl radical ($\cdot OH$) (Todoki et al., 1992; Prasad and Bharadwaj 1996).

The four concentrations of DHF used in these studies were termed 1X, 2X, 4X, and 8X as follows:

(1X)-DHF (6.25 μg/ml), $FeCl_3$ (5.0 μg/ml)and ADP(81.3 μg/ml)

(2X)-DHF (12.5 μg/ml), $FeCl_3$ (10 μg/ml) and ADP (163 μg/ml)

(4X)-DHF (25 μg/ml), $FeCl_3$ (20 μg/ml) and ADP (325 μg/ml)

(8X)-DHF (50 μg/ml), $FeCl_3$ (40 μg/ml) and ADP (650 μg/ml)

Hydroxyl radical was produced by the following reaction:

$$DHF + O_2 \text{ ------> } [DHF]^{\cdot} + O_2^{\cdot -} \qquad [4.2]$$

$$2H^{+} + O_2^{\cdot -} + DHF \text{ ------> } [DHF]^{\cdot} + H_2O_2 \qquad [4.3]$$

$O_2^{\cdot -}$ + Fe^{3+}-ADP -----> O_2 + Fe^{2+}-ADP [4.4]

Fe^{2+}-ADP + H_2O_2 -----> Fe^{3+}-ADP + $\cdot OH$ + OH- [4.5]

All solutions were made in Krebs-Ringer solution and 0.1 to 0.4 ml of solutions were added in the tissue bath to elicit responses. Note: The use of the concentrations (1X, 2X, 4X, and 8X) of dihydroxy fumaric acid, ferric chloride and adenosine diphosphate are represented by the abbreviation DHF.

4.2.3 Hydrogen Peroxide Challenge

In this study, three different concentrations of hydrogen peroxide (0.1, 0.2, 0.4 mM) were used. Hydrogen peroxide was added directly to the organ bath to elicit responses.

4.3 Chemicals and Drugs

4.3.1 Acetylcholine

Acetylcholine (ACh) is released in the body by cholinergic fibers and binds to the acetylcholine receptor (cholinoceptor). The enzyme acetylcholinesterase very efficiently splits acetylcholine into choline and acetate, neither of which has significant transmitter potency; therefore, its effects are short lived and local.

The dose of acetylcholine (0.5 μg/ml) used in this study before and after treatment of tissues with free radicals was chosen based on the result of the preliminary study, to

produce submaximal contraction of ileum.

4.3.2 Antioxidants

4.3.2.1 Superoxide Dismutase

Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 at a rate 10,000 times faster than spontaneous dismutation at physiologic pH (Fridovich, 1983). While lower concentrations of SOD are effective in ameliorating free radical-mediated tissue damage, higher doses may actually exacerbate the injury, possibly by "overscavenging" superoxide and thus inhibiting the termination step of lipid peroxidation (Reilly et al., 1991). Superoxide dismutase is rapidly excreted by the kidneys and has a circulatory half-life of less than 10 minutes.

Superoxide dismutase was obtained from the Sigma Chemical Company as a 4,200 U/mg protein, and a concentration of 100 U/ml SOD was used in this study.

4.3.2.2 Catalase

Catalase, a membrane-impermeable antioxidant, enzymatically converts H_2O_2 into oxygen and water. Catalase has a half-life of approximately 20 minutes *in vivo* and is degraded by proteolysis. The large molecular weight of catalase necessitates modification to allow it entry into cells (Reilly et al., 1991).

Catalase (11,000 U/mg protein) was purchased from the

Sigma Chemical Company. The concentration of catalase (500 U/ml) used in this study was based on the results obtained from previous experiments with trachea (Gupta and Prasad, 1992).

4.3.2.3 Mannitol

Mannitol, a non-enzymatic hydroxyl radical scavenger by virtue of its many hydroxyl groups, can scavenge a hydroxyl radical to form a mannitol free radical. This radical then preferentially reacts with another mannitol radical to form a stable non-reactive mannitol dimer (Reilly et al., 1991).

The concentrations of mannitol used in this study were 40, 80, and 160 mM.

4.3.2.4 Dimethylthiourea

Dimethylthiourea (DMTU) is a powerful scavenger of hydroxyl radical, hydrogen peroxide and hypochlorous acid. Dimethylthiourea is also highly diffusible across lipid membranes, allowing it to act intra-cellularly as well (Reilly et al., 1991).

Three different concentrations of DMTU (36.3 μg/ml, 72.6 μg/ml, and 1.6 mg/ml) were utilized in this study.

4.3.2.5 Histidine

Histidine is an excellent quencher of singlet oxygen ($^1O_2$) and has a high rate constant for its interaction with

$^1O_2$ ($k = 1 \times 10^8\ MS^{-1}$). It appears to protect by scavenging $^1O_2$, which is the end product of free radical chain reactions and can directly inactivate proteins. When histidine reacts with $^1O_2$, it forms an endoperoxide which then decomposes to a complex mixture of non-reactive products (Kukreja and Hess, 1992).

Two concentrations of histidine (100 and 200 mM) were used in this study.

4.3.2.6 Deferoxamine

Deferoxamine is a siderophore isolated from Streptomyces pilosus, which binds 1 molecule of Fe^{3+} per molecule of deferoxamine. Deferoxamine completely "locks in" the iron ion in a 1:1 complex, binding to all of the six coordination sites (Asbeck, 1990), thus inhibiting iron catalysis of the Haber-Weiss reaction. In addition to the inhibition of radical formation by chelation of iron, deferoxamine may inhibit oxidant damage by directly scavenging $\cdot OH$, and by the inhibition of peroxidase-catalyzed reactions. Another antioxidant feature of deferoxamine is its capacity to react with superoxide, forming a relatively stable nitroxide. The reaction of deferoxamine with $O_2\cdot^-$, however, is very slow (Asbeck, 1990).

High concentrations of deferoxamine were used in this study, (50 and 100 mM), because it is much more effective in preventing the formation of hydroxyl radicals than in

scavenging them after they are formed (Reilly et al., 1991).

4.3.3 Specific Mediator Inhibitors

4.3.3.1 Indomethacin

Indomethacin is an inhibitor of cyclooxygenase. The antiinflammatory activity of indomethacin could be due in part to its antioxidant property; however, the antioxidant activity of indomethacin occurs at a concentration higher than that used for cyclooxygenase inhibition (Prasad and Laxdal, 1994). Indomethacin is rapidly absorbed from the gastrointestinal tract following its oral ingestion. The half-life in plasma is variable and ranges between 2 and 11 hours.

Three concentrations of indomethacin (10^{-5}, $2x10^{-5}$, $4x10^{-5}$ M) were used in this study.

4.3.3.2 N^G-monomethyl-L-arginine

N^G-monomethyl-L-arginine (L-NMMA) inhibits the enzyme nitric oxide synthase. Nitric oxide synthase oxidizes the guanidine nitrogen of L-arginine to yield nitric oxide (Palmer et al., 1988). Previous work with aorta and airway smooth muscle has shown that L-NMMA reduces the effects of OFRs (Bharadwaj and Prasad, 1997; Gupta and Prasad, 1992).

The concentration of L-NMMA (0.25 mM) used in this study was similar to that previously used by Bharadwaj and Prasad, (1997).

4.3.3.3 Methylene Blue

Methylene blue is a dye that has been used as a vital stain for nervous tissue. It is also a specific inhibitor of the soluble guanylate cyclase (Ignarro and Kadowitz, 1985). Methylene blue has been shown to reduce the effects of oxygen free radicals on airway smooth muscle and aorta (Gupta and Prasad, 1992; Bharadwaj and Prasad, 1997).

The concentration of methylene blue (10^{-5} M) used in this study was based on the results obtained from studies on aorta and trachea (Gupta and Prasad, 1992; Bharadwaj and Prasad, 1997).

4.3.3.4 Glibenclamide

The sulfonylurea, glibenclamide, is a potent and selective inhibitor of ATP-sensitive K^+ channels. It has effectively blocks the *in vitro* relaxation of ileum by some potassium channel openers. This drug also blocks contractions induced by prostaglandin $F_2\alpha$, and prostaglandin E_2 (Delaey and Van de Voorde, 1995). Glibenclamide is absorbed relatively rapidly from the gastrointestinal tract, and peak concentrations in plasma have been detected in 4 hours. The half-life of glibenclamide in plasma is about 10 hours (Hardman et al., 1995).

The concentration of glibenclamide (10^{-5} M) used in this study was similar to that used by Bharadwaj and Prasad, (1997).

4.3.3.5 Atropine

Atropine sulphate is an antimuscarinic drug, clinically used to block adverse muscarinic effects of anticholinesterase agents. It is well absorbed from the gut with a half-life of 2 hours. Atropine causes reversible competitive blockade of the actions of acetylcholine at muscarinic receptors. The result of binding to the muscarinic receptor is the prevention of the release of inositol triphosphate (IP_3) and the inhibition of adenyl cyclase that is brought by acetylcholine. Atropine also modifies neuromuscular transmission and increased transmitter release, possibly by inhibiting presynaptic muscarinic receptors. Such receptors are well characterized in the central nervous system and on peripheral smooth muscle (Wali et al., 1987). The concentration of atropine used in this study was 10^{-6} M.

4.3.3.6 Pyrilamine

Pyrilamine , a histamine-receptor antagonist, blocks the action of histamine by reversible competitive antagonism at the H_1 receptor. Pyrilamine is rapidly absorbed following oral administration, with peak blood concentration occurring in 1-2 hours.

The concentrations of pyrilamine used in this study were 10^{-5} and 10^{-4} M.

4.4 Protocol

4.4.1 Xanthine plus Xanthine Oxidase (X/XO)

4.4.1.1 Reactive Oxygen Metabolites Responsible for Change in Ileum Motility

Animals were divided into different groups, and the number of animals which used in each group was 6 (n=6).

Group I: The effects of three concentrations (1, 2, and 4X) of X/XO on the basal tone of the ileum preparation were studied for 20 minutes.

Group 11: To investigate the influence of oxidants generated by X/XO on ileal smooth muscle receptors and tissue integrity, the contractile response to acetylcholine was recorded before and after 20 minutes of incubation of the tissue with X/XO.

Group III: This study was designed to examine whether the effects of X/XO were mediated through the production of superoxide anion ($O_2^{\cdot -}$). The effects of three concentrations of X/XO were examined in the presence and absence of SOD (a superoxide anion metabolizer). Superoxide dismutase was added to the organ bath containing the ileum tissues 3 minutes prior to the addition of X/XO. The effects were monitored for 20 minutes.

Group IV: To assess the relative role of hydrogen peroxide in the response induced by X/XO, catalase was used. Catalase was added to the bath 3 minutes before addition of X/XO. The effects were monitored for 20 minutes.

Group V: To examine if the effects of X/XO were mediated through hydroxyl radical formation, the effects of three concentrations of X/XO in the presence and absence of various concentrations of dimethylthiourea and mannitol (hydroxyl radical scavengers) on the basal tone were evaluated for 20 minutes. These scavengers were added to the bath 20 minutes before addition of X/XO.

Group VI: To evaluate the role of iron in the X/XO-induced relaxation, the iron chelator deferoxamine, which will prevent the Haber-Weiss reaction, was used. The effect of 2X concentration of X/XO in the presence and absence of deferoxamine were investigated for a period of 20 minutes. Deferoxamine was added to the bath 20 minutes before addition of X/XO.

Group VII: The effects of X/XO in the presence and absence of histidine ($^1O_2$ scavenger) were examined for 20 minutes to determine if the effects are mediated through the formation of singlet oxygen ($^1O_2$). Histidine was added to the organ bath 20 minutes prior to addition of X/XO.

4.4.1.2 Mechanisms of Xanthine plus Xanthine Oxidase-Induced Relaxation

Group I: This study was designed to determine the role of arachidonic acid metabolites. The effects of three concentrations of X/XO in the absence and presence of increasing concentrations of indomethacin were measured.

Indomethacin was added to the bath 20 minutes prior to addition of X/XO.

Group II: The role of nitric oxide (NO) in X/XO-induced relaxation of ileum preparation was studied in this group. N^G-monomethyl-L-arginine (L-NMMA) inhibits the enzyme nitric oxide synthase. Therefore, the effects of three concentrations of X/XO in the presence and absence of L-NMMA were monitored over a period of 20 minutes. L-NMMA was added to the bath 20 minutes before addition of X/XO.

Group III: This investigation evaluated the role of guanosine 3',5'-cyclic monophosphate (cGMP) in the X/XO modulation of ileum tone. The effects of three concentrations of X/XO were investigated in the presence and absence of methylene blue over a period of 20 minutes. Methylene blue inhibits cGMP synthesis. It was added to the bath 20 minutes prior to addition of X/XO.

Group IV: To rule out the role of ATP-sensitive K^+ channels in the X/XO-induced modulation of ileum tone, the effects of three concentrations of X/XO in the presence and absence of glibenclamide, an inhibitor of ATP-sensitive K^+ channel, were examined for 20 minutes. Glibenclamide was added to the bath 20 minutes before addition of X/XO.

4.4.2 Hydrogen Peroxide (H_2O_2)

4.4.2.1 Reactive Oxygen Metabolites Responsible for Change in Ileum Motility

Group I: The effects of three concentrations (1,2, and 4X) of H_2O_2 were studied on the basal tone of the ileum preparation for 20 minutes.

Group II: This study was designed to determine if the effects of H_2O_2 were mediated through hydroxyl radical formation. The effects of three different concentrations of H_2O_2 were evaluated in the presence and absence of $\cdot OH$ scavengers (dimethylthiourea and mannitol) for 20 minutes. Hydroxyl radical scavengers were added to the bath 20 minutes prior to addition of H_2O_2.

4.4.2.2 Mechanisms of Hydrogen Peroxide-Induced Responses

Group I: To evaluate the role of arachidonic acid metabolites in the H_2O_2-induced modulation of ileum tone, the effects of three different concentrations of H_2O_2 were observed in the absence and presence of increasing concentrations of indomethacin for over a period of 20 minutes. Indomethacin was added to the organ bath 20 minutes before addition of H_2O_2.

Group II: This study was designed to determine whether or not H_2O_2-induced relaxation of ileum tone is mediated through nitric oxide. Effects of H_2O_2 in the presence and absence of L-NMMA were measured. L-NMMA was added to the bath 20 minutes prior to addition of H_2O_2.

Group III: This investigation evaluated the role of guanosine 3',5'-cyclic monophosphate (cGMP) in the H_2O_2-relaxation of ileum tone. The effects of H_2O_2 were investigated in the presence and absence of methylene blue for 20 minutes. Methylene blue inhibits cGMP synthesis. It was added to the bath 20 minutes prior to addition of H_2O_2.

Group IV: This study was undertaken to determine the role of ATP-sensitive K^+ channel in the H_2O_2-induced modulation of ileum tone. Therefore, the effects of H_2O_2 in the presence and absence of glibenclamide were observed for 20 minutes. Glibenclamide was added to the organ bath 20 minutes before addition of H_2O_2.

Group V: The effects of H_2O_2 in the presence and absence of atropine were studied for 20 minutes to determine the role of acetylcholine in the H_2O_2-induced modulation of ileum tone. Atropine was added to the bath 20 minutes prior to the addition of H_2O_2.

Group VI: This study was designed to determine the role of histamine. The effects of H_2O_2 in the presence and absence of pyrilamine (H_1-receptor antagonist) were examined over a period of 20 minutes. Pyrilamine was added to the bath 20 minutes before addition of H_2O_2.

4.4.3 Dihydroxy Fumaric Acid (DHF) plus Ferric Chloride ($FeCl_3$) and Adenosine Diphosphate (ADP)

4.4.3.1 Reactive Oxygen Metabolites Responsible for Change in

Ileum Motility

Group I: The effects of four concentrations (1, 2, 4, and 8X) of DHF on the basal tone of ileum preparation were studied for 20 minutes.

Group II: To assess the role of superoxide anion ($O_2^{\cdot -}$) in the DHF-induced responses, the effects of various concentrations of DHF in the presence and absence of SOD (a $O_2^{\cdot -}$ metabolizer) on the basal tone of ileum were examined. Superoxide dismutase was added to the organ bath 3 minutes prior to addition of DHF. The effects were evaluated for 20 minutes.

Group III: To evaluate if the effects of DHF were mediated through the formation of hydrogen peroxide (H_2O_2), the effects of DHF in the presence and absence of catalase (H_2O_2 metabolizer) were evaluated for 20 minutes. Catalase was added to the bath 3 minutes before addition of DHF.

Group IV: This study was undertaken to determine if the effects of DHF were mediated through the hydroxyl radical ($\cdot OH$). The effects of various concentrations of DHF in the presence and absence of three increasing concentrations of dimethylthiourea and mannitol ($\cdot OH$ scavengers) were examined for 20 minutes. The scavengers were added to the organ bath 20 minutes prior to addition of DHF.

Group V: To find if the effects of DHF were mediated through the production of singlet oxygen ($^1O_2$), the effects of DHF in the presence and absence of histidine ($^1O_2$

scavenger) were evaluated for 20 minutes. Histidine was added to the bath 20 minutes before addition of DHF.

4.4.3.2 Mechanisms of Dihydroxy Fumaric Acid plus Ferric Chloride and Adenosine Diphosphate-Induced Contraction

Group I: The effects of various concentrations of DHF in the absence and presence of increasing concentrations of indomethacin were observed for 20 minutes. This study was performed to determine if the effects of DHF were mediated by arachidonic acid metabolites. Indomethacin was added to the bath 20 minutes prior to addition of DHF.

Group II: The effects of DHF in the presence and absence of atropine were investigated over 20 minutes to determine the role of acetylcholine and muscarinic receptors in DHF-induced modulation of ileum tone. Atropine was added to the bath 20 minutes prior to addition of DHF.

Group III: The effects of pyrilamine on the DHF-induced contraction were investigated for 20 minutes to see if the effects were mediated through the release of histamine. Pyrilamine was added to the bath 20 minutes before addition of DHF.

4.5 Statistical Analysis

The results were expressed as mean ± standard error (± S.E., n=6). The data were analyzed using paired and unpaired student's t-test. P values less than 0.05 were considered statistically significant.

5.0 RESULTS

5.1 Effects of Xanthine plus Xanthine Oxidase (X/XO) on Ileum Preparations

The effects of three concentrations of xanthine plus xanthine oxidase on the baseline tension of ileum preparations were observed for 20 minutes. The summary of results of the three concentrations of X/XO are shown in Figure 1. Xanthine plus xanthine oxidase produced relaxation of ileum preparations. The response of tissue to exogenous addition of X/XO was a biphasic, with an initial relaxation occurring at approximately 1 minute followed by a second relaxation maximum at 20 minutes. Relaxation was concentration dependent up to 2X concentration of X/XO which the relaxation with 4X was smaller than with the 2X concentration. The resting tension returned to baseline after repeated washing, and the time required for ileum strips to fully return to baseline was roughly 15-30 minutes post-exposure to X/XO.

Experiments were also conducted to determine whether or not the contractile responses to acetylcholine (ACh) were affected by the presence of X/XO. The contractile response to ACh (0.5 μg/ml) was tested before and after treatment of tissues with X/XO. The results of ACh response in absence and

presence of three concentrations of X/XO are summarized in Figure 2. The responses to ACh following X/XO exposure were greater for 1X, and 2X; and smaller in magnitude for 4X than the maximum initial ACh contraction.

5.1.1 Oxygen Metabolites Generated by X/XO System

Following the addition of xanthine to xanthine oxidase, the enzyme generates superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2)(Porras et al., 1981). At neutral pH, approximately 20 % of the total electron flux through the oxidase can be accounted for as the univalent product, $O_2^{\cdot -}$. Although the xanthine-xanthine oxidase system can generate both $O_2^{\cdot -}$ and H_2O_2, Beauchamp and Fridovich (1970) demonstrated that the two species can interact via the process known as the Haber-Weiss reaction to generate the hydroxyl radical ($\cdot OH$). Furthermore, singlet oxygen ($^1O_2$) can be generated by the reaction of the xanthine oxidase enzyme (Kukreja and Hess, 1992). Therefore, following activation of the xanthine plus xanthine oxidase system a complex spectrum of oxidants ($O_2^{\cdot -}$, H_2O_2, $\cdot OH$, and $^1O_2$) can be generated and the ability to predict the "final" oxidant is difficult. In the following sections the effects of X/XO in the absence and presence of different antioxidants were be evaluated to find which free radical is involved in the relaxation of ileum.

5.1.1.1 Effect of Superoxide Dismutase on X/XO-Induced Relaxation of Ileum

To assess the relative role of $O_2^{\cdot -}$ in the X/XO-induced relaxation, the antioxidant, SOD was used in this study. If the $O_2^{\cdot -}$ produces relaxation of isolated ileum strips, the relaxant effect would be abolished in the presence of SOD, which should metabolize $O_2^{\cdot -}$ to H_2O_2. Therefore, all strips were pretreated with SOD for 2-3 minutes prior to the addition of X/XO.

The effects of 2X concentration of X/XO in the absence and presence of 100 U/ml SOD were observed for a 20 minute period, and the results are summarized in Figure 3. The results of 1X and 4X concentrations of X/XO summarized in Appendix (Figure 1). The pretreatment with SOD did not prevent the relaxing effect of X/XO. This result suggests that X/XO-induced relaxation is not mediated through the superoxide anion.

5.1.1.2 Effect of Catalase on X/XO-Induced Relaxation of Ileum

Catalase was used in this study in order to establish if the X/XO effects are mediated through H_2O_2. If H_2O_2 was the oxygen free radical responsible for the change in tension, the response would be diminished by the exogenous addition of catalase, which rapidly degrades H_2O_2 to O_2 and H_2O. Catalase was added 5 minutes prior to addition of X/XO. The summary of

the effects of 2X concentration of X/XO on the resting tension in the absence and presence of catalase (500 U/ml) are shown in Figure 3, and the results of 1X and 4X concentrations of X/XO summarized in the Appendix (Figure 1). The tension developed in the presence of catalase was not significantly different from that developed in its absence.

5.1.1.3 Effect of Superoxide Dismutase plus Catalase on X/XO-Induced Relaxation

Superoxide dismutase and catalase were used in this investigation to eliminate the effects of both $O_2^{\cdot -}$ and H_2O_2 in the relaxation induced by X/XO. The results of 2X concentration of X/XO in the absence and presence of SOD (100 U/ml) and catalase (500 U/ml) are summarized in Figure 3, and the results of 1X and 4X concentrations of X/XO summarized in the Appendix (Figure 1). There were no significant differences in X/XO-induced relaxation in the absence or presence of SOD + CAT, suggesting that neither $O_2^{\cdot -}$ nor H_2O_2 are involved in the relaxation induced by X/XO.

5.1.1.4 Effect of Dimethylthiourea on X/XO-Induced Relaxation of Ileum

Hydroxyl radicals ($^{\cdot}OH$) generated from superoxide anion have also been implicated as toxic products formed by the X/XO system (Fridovich, 1975). Hydrogen peroxide, as another by product of X/XO system, readily penetrates cell membranes,

enters the cells and reacts with intracellular iron to form hydroxyl radicals. Therefore, the hydroxyl radical scavenger DMTU was used to determine whether or not the ·OH was responsible for X/XO-induced relaxation of ileum. Dimethylthiourea was added 15-20 minutes before addition of X/XO. High concentrations of DMTU were used because the site of hydroxyl radical generation is not clear, and hydroxyl radical has high reactivity, reacting with molecules within a 14 A° ranges in a period of less than 10^{-6} seconds. Therefore, for an ·OH scavenger to be effective, it must be present in concentrations so that it will comprise a significant proportion of the total molecules.

The effects of 1X, 2X, and 4X concentrations of X/XO in the absence and presence of 1.6 mg/ml DMTU are summarized in the Appendix (Figure 2), Figure 4 and the Appendix (Figure 2) respectively. The results show that pretreatment with DMTU reduced the initial maximum relaxation by 56.34% (i.e., from 42.57 to 18.58%) for 1X, 50.09% (i.e., from 69.02 to 34.44%) for 2X, and 57.85% (i.e., from 63.67 to 26.83%) for 4X. The second maximum relaxation was reduced by 67.08% (i.e., from 56.93 to 18.74%) for 1X, 51.07% (i.e., from 62.06 to 30.36%) for 2X, and 55.33% (i.e., from 46.24 to 20.65%) for 4X. Also in the presence of DMTU, the time-frame of peak tension was similar to controls (X/XO). DMTU alone produced small relaxation which was maximal at 15 minutes after which it remained constant. These results suggest that X/XO-induced

relaxation is mediated through the hydroxyl radical.

5.1.1.5 Effect of Mannitol on X/XO-Induced Relaxation of Ileum

To further ascertain the role of hydroxyl radical in X/XO-induced relaxation of ileum, another scavenger of hydroxyl radical, mannitol, was studied. Figure 5 summarizes the effects of 2X concentration of X/XO on rat ileum in the absence and presence of mannitol (80 mM).

Mannitol by itself produced small contraction after 5 minutes which remained constant during 40 minutes of treatment. There was a significant difference between control (X/XO alone) and mannitol-treated groups. The effects of 1X and 4X concentrations of X/XO in the absence and presence of mannitol are summarized in the Appendix (Figure 2). Pretreatment with mannitol reduced the initial maximum relaxation by 59.34% (i.e., from 42.57 to 17.31%) for 1X, 39.76% (i.e., from 69.02 to 41.57%) for 2X, and 40.26% (i.e., from 63.67 to 38.03%) for 4X. The second maximum relaxation of was reduced by 50.68% (i.e., from 56.93 to 28.07%) for 1X, 43.2% (i.e., from 62.06 to 35.24%) for 2X, and 41.87% (i.e., from 46.24 to 26.87%) for 4X. These results suggest that the relaxation is mediated through the hydroxyl radical.

5.1.1.6 Effect of Deferoxamine on X/XO-Induced Relaxation of Ileum

Pretreatment with DMTU and mannitol showed that the hydroxyl radical is involved in the relaxation induced by X/XO. However, the formation of hydroxyl radical from superoxide and hydrogen peroxide requires the presence of iron. Green and Mazur (1957) noted that the xanthine oxidase reaction caused release of iron from ferritin (the primary intracellular storage form of iron) which was not inhibited by catalase. Subsequent availability of SOD showed that $O_2^{\cdot -}$ produced by the XO was responsible for the mobilization of iron (Fridovich, 1988). Therefore, to assess whether intracellular iron plays a role in relaxation induced by X/XO, the iron chelator, deferoxamine, which will prevent the Haber-Weiss reaction, was used. The tissues were pretreated with deferoxamine (50 or 100 mM) 20 minutes prior to addition of X/XO. The effects of 2X concentration of X/XO in the absence and presence of deferoxamine are summarized in Figure 6.

Deferoxamine significantly reduced the relaxation induced by X/XO, and this effect was concentration-dependent. Deferoxamine alone produced a small contraction of tissues during the experiments. In the presence of deferoxamine (50 mM) ileum tension was not significantly different from control groups (X/XO) in the first 10 minutes; however, the tension was significantly different at 15 and 20 minutes.

Pretreatment with deferoxamine (50 and 100 mM) reduced the initial relaxation by 24.78% and 91.48%, and the second relaxation by 37.35% and 88.16%, respectively. This finding also suggests that hydroxyl radical may be involved the relaxation induced by X/XO.

5.1.1.7 Effect of Histidine on X/XO-Induced Relaxation of Ileum

Singlet oxygen ($^1O_2$) generation has been suggested as a toxic agent that can be generated from superoxide anion in the xanthine/xanthine oxidase system (Duran, 1982). Spontaneous dismutation of $O_2^{\cdot -}$ can also generate singlet oxygen. Thus, it is possible that $^1O_2$ may play a role in X/XO-induced relaxation of ileum. The effects of histidine (a $^1O_2$ scavenger) on X/XO-induced relaxation of ileum were therefore investigated. The effects of xanthine plus xanthine oxidase in the absence and presence of two concentrations of histidine on ileum preparations are summarized in Figure 7.

Xanthine plus xanthine oxidase produced relaxation of ileum as expected. Pretreatment with histidine significantly reduced the relaxation, and this reduction was concentration-dependent. Pretreatment with 100 and 200 mM histidine reduced the initial maximum relaxation by 38.4% and 87.26%, and subsequent maximum relaxation by 29.56% and 87.81%, respectively. These results suggest that $^1O_2$ also plays a role in X/XO-induced relaxation.

5.1.2 Mechanisms of X/XO-Induced Relaxation

5.1.2.1 Effect of Indomethacin on X/XO-Induced Relaxation of Ileum

Oxidants generated by xanthine and xanthine oxidase have been shown to increase PGE_2 and prostacyclin tissue levels (Bern et al., 1989; Karayalcin et al., 1990). Chakraborti et al., (1989) have reported that oxidants increased phospholipase A_2 activity, which in turn increased the release of arachidonic acid and synthesis of PGE_2 and PGI_2. These compounds cause contraction and relaxation of smooth muscle, respectively. Therefore, the effects of X/XO in the absence and presence of indomethacin (10^{-5}, 2 x 10^{-5}, and 4 x 10^{-5} M) on ileum preparations were examined to determine if arachidonic acid metabolites are involved in X/XO-induced relaxation.

The effects of 2X concentration of X/XO in the absence and presence of various concentrations of indomethacin are summarized in Figure 8. The results show that there was a significant difference between control (X/XO) and indomethacin-treated groups, however, there was no significant difference between different groups treated with concentrations of indomethacin (10^{-5}, $2x10^{-5}$, and $4x10^{-5}$ M). Pretreatment with indomethacin (10^{-5}, $2x10^{-5}$, $4x10^{-5}$ M) reduced the initial relaxation by 53.95%, 54.40% and 58.29% respectively. The subsequent maximum relaxation was reduced by 51.75%, 62.47% and 64.1%, respectively, by indomethacin

(10^{-5}, $2x10^{-5}$, $4x10^{-5}$ M). The results of the effects of 1X and 4X concentrations of X/XO summarized in the Appendix (Figure 3).

These results suggest that arachidonic acid metabolites are involved in X/XO-induced relaxation of ileum.

5.1.2.2 Effect of N^G-monomethyl-L-arginine on X/XO-Induced Relaxation of Ileum

N^G-monomethyl-L-arginine, an inhibitor of the enzyme nitric oxide synthase, was used to establish if X/XO-induced relaxation of ileum is mediated through the nitric oxide pathway. The effects of 2X concentration of X/XO in the absence and presence of L-NMMA are summarized in Figure 9 and the results of 1X and 4X of X/XO are summarized in the Appendix (Figure 4). Pretreatment with L-NMMA (0.25 mM) did not have any effect on relaxation induced by X/XO; however, there was a significant difference between the X/XO and the group treated with L-NMMA (1 mM) at 5 minutes. Pretreatment with L-NMMA (1mM) reduced the relaxation by 31.72% (i.e., from 49.62 to 33.88%). There were no significant differences in the responses of ileum to X/XO with or without L-NMMA at other times. L-NMMA alone did not have any effect on tissues. These results suggest a partial involvement of nitric oxide in X/XO-induced relaxation of ileum preparations.

5.1.2.3 Effect of Methylene Blue on X/XO-Induced Relaxation of Ileum

Methylene blue was used to assess the role of cGMP in X/XO-induced relaxation of ileum. The effects of 2X concentration of X/XO in the absence and presence of methylene blue (10^{-5} M) on ileum are summarized in Figure 10 and the results of 1X and 4X of X/XO summarized in the Appendix (Figure 4). Pretreatment with methylene blue did not significantly reduce the relaxant effect of X/XO.

5.1.2.4 Effect of Glibenclamide on X/XO-Induced Relaxation of Ileum

Glibenclamide, an inhibitor of ATP-sensitive potassium channels, was used to investigate the possible role of the ATP-sensitive K^+ channel in X/XO-induced relaxation. The effects of 2X concentration of X/XO in the absence and presence of various concentrations of glibenclamide are summarized in Figure 11 and the results of 1X and 4X concentrations of X/XO are summarized in the Appendix (Figure 5). Glibenclamide alone produced a small relaxation of tissues which remained constant during the experiments. There were no significant differences between control (X/XO) and groups treated with glibenclamide ($2x10^{-5}$ M); however, in the presence of $4x10^{-5}$ and $8x10^{-5}$ M glibenclamide relaxation were significantly different from control groups (2X and 4X concentrations of X/XO) at times 3, 5, 10, and 15 minutes.

5.1.3 Effect of X/XO on Spontaneous Activity of Ileum

In the absence of any stimulation, the ileum preparations exhibited spontaneous contraction. When the ileum was treated with X/XO, the magnitude of spontaneous activity suddenly dropped, followed by a slight recovery, and then gradually decreased. The results of amplitude of spontaneous activity in absence and presence of three concentrations (1X, 2X, and 4X) of X/XO are summarized in Figure 12. In the presence of X/XO, the magnitude of spontaneous activity of ileum decreased in a concentration-dependent manner. Xanthine/xanthine oxidase reduced the amplitude of initial relaxation to 34.86 % for 1X, 50.05 % for 2X and 18.24 % for 4X concentrations. The amplitude of subsequent relaxation was 16.66 % for 1X, 13.02 % for 2X and 3.9 % for 4X respectively.

To elucidate the mechanism of inhibition of amplitude of spontaneous activity with X/XO, indomethacin was used to determine the possible contribution of arachidonic acid metabolites. The results of spontaneous activity in the absence and presence of 2X concentration of X/XO, and increasing concentrations of indomethacin are summarized in Figure 13; and the results of 1X and 4X concentrations of X/XO are summarized in the Appendix (Figure 6). Indomethacin alone did not affect the spontaneous activity of the ileum. Xanthine plus xanthine oxidase decreased the spontaneous activity, as described before. There were significant

differences between control (X/XO) and groups treated with indomethacin. Pretreatment with indomethacin ($2x10^{-5}$ and $4x10^{-5}$ M) prevented the X/XO-induced decrease in maximal amplitude of spontaneous activity by 60.65 % (i.e., from 14.62 to 37.16%) and 83.71% (i.e., from 14.62 to 89.77 %) and the minimal amplitude of spontaneous activity by 63.06 % (from 13.02 to 35.25%) and 78.90 % (from 13.02 to 61.73 %), respectively. These results suggest that arachidonic acid metabolites are involved in the X/XO-induced decrease of the amplitude of spontaneous activity of ileum.

5.2 Effects of Hydrogen Peroxide (H_2O_2) on Ileum Preparations

Effects of three concentrations of H_2O_2 (1X, 2X, and 4X) on the ileum preparations were observed for 20 minutes, and the results are summarized in Figure 14.

Hydrogen peroxide produced a biphasic response consisting of an initial small contraction followed by a significant relaxation that reached to a maximum value at 10 minutes. The contraction induced by H_2O_2 was of short duration. The peak of contraction was at 2.5-3 minutes post-addition of H_2O_2. The mean responses were observed to increase as concentration of H_2O_2 increased. The maximum relaxing response was elicited by the 4X concentration of H_2O_2. Treatment with H_2O_2 produced maximum contraction of 17.86, 21.50, and 23.57 % and maximum relaxation of 17.87, 20.79, and 30.64% for 1X, 2X, and 4X concentrations of H_2O_2,

respectively.

5.2.1 Effects of H_2O_2 in the Presence of Catalase

Catalase, a metabolizer of H_2O_2, was used to investigate if the H_2O_2 effects are blocked. The effects of 2X concentration of H_2O_2 on the basal tension of ileum preparations in the absence and presence of catalase (500 U/ml) are summarized in Figure 15 and the results of 1X and 4X concentrations of H_2O_2 summarized in the Appendix (Figure 7). Catalase alone did not affect the basal tone. However, pretreatment with catalase (500 U/ml) significantly reduced the contractile effect of 2X concentration of H_2O_2, and it completely inhibited the relaxant effect of H_2O_2. The mean contractile response to the sole addition of H_2O_2 was 21.50%; however, pretreatment with catalase resulted in a contractile response of 7.04%. This result suggests that the hydrogen peroxide-induced biphasic response is mediated through H_2O_2.

5.2.2 Effects of H_2O_2 in the Presence of Dimethylthiourea or Mannitol

Because H_2O_2 can readily penetrate cell membranes it is conceivable that H_2O_2 enters the cells and reacts with intracellular iron to form hydroxyl radical. Therefore, dimethylthiourea and mannitol , two hydroxyl radical scavengers, were used to establish whether or not the effects of H_2O_2 also involved $\cdot OH$. To achieve this objective, effects

of three concentrations of H_2O_2 on the ileum in the absence and presence of two hydroxyl radical scavengers were investigated.

Figures 16 and 17 summarize the results obtained in the absence and presence of DMTU (1.6 mg/ml) and mannitol (80 and 160 mM). The results of 1X and 4X concentrations of H_2O_2 are summarized in the Appendix (Figure 7). Dimethylthiourea inhibited contractile response and blocked the relaxant effects of H_2O_2, (61.34 and 79.41%) at 2X concentration of H_2O_2; however, in the presence of mannitol, the contractile effect was completely abolished. Also, significant differences between control (H_2O_2) and mannitol-treated groups were observed for the relaxant effect of H_2O_2. Pretreatment with mannitol (80 and 160 mM) reduced the relaxant effect of H_2O_2 by 30.15 % (i.e., from 20.79 to 14.52 %) and 75% (i.e., from 20.79 to 5.28 %), respectively. These results indicate that both contraction and relaxation induced by H_2O_2 are also mediated by ·OH.

5.2.3 Mechanisms of H_2O_2-Induced Biphasic Responses

5.2.3.1 Effects of H_2O_2 in the Presence of Indomethacin

Indomethacin was used to determine if the H_2O_2-induced biphasic response is mediated through arachidonic acid metabolites. The effects of H_2O_2 in the absence and presence of two concentrations of indomethacin are summarized in Figure 18. The results of 1X and 4X concentrations of H_2O_2

summarized in the Appendix (Figure 8). As expected, 2X concentration of H_2O_2 produced a biphasic response. Indomethacin at all concentrations reduced both contraction and relaxation significantly, indicating that the effects of H_2O_2 are partially mediated by the release of arachidonic metabolites. Pretreatment with indomethacin ($2x10^{-5}$ and $4x10^{-5}$ M) reduced the contraction of 2X H_2O_2 by 52.18 % (i.e., from 21.50 to 10.28 %) and 84.13 % (i.e., from 21.50 to 3.41 %) and reduced the maximum relaxation by 6.68% (i.e., from 20.79 to 19.4 %) and 58.82 % (i.e., from 20.79 to 8.56 %), respectively.

5.2.3.2 Effects of H_2O_2 in the Presence of Glibenclamide

The effects of H_2O_2 in the absence and presence of glibenclamide (10^{-5} M) on ileum preparations are summarized in Figure 19. The results of 1X and 4X concentrations of H_2O_2 summarized in the Appendix (Figure 9). Glibenclamide was used to establish the possible role of ATP-sensitive K^+ channels in H_2O_2-induced relaxation. The relaxant effect of H_2O_2 was not reduced by glibenclamide; however, the contractile effect of H_2O_2 on ileum was significantly reduced by 61.53% (i.e., from 21.5 to 8.27%) at 2X H_2O_2 in the presence of glibenclamide, suggesting a role of prostaglandins in H_2O_2-induced contraction of ileum.

5.2.3.3 Effects of H_2O_2 in the Presence of N^G monomethyl-L-arginine

N^G-monomethyl-L-arginine (L-NMMA) inhibits the synthesis of nitric oxide by antagonizing the enzyme nitric oxide synthase. L-NMMA was therefore used to establish whether or not the H_2O_2-induced relaxant effect is mediated through NO. The effects of 2X concentration of H_2O_2 in the absence and presence of L-NMMA (0.25 mM) were monitored over a period of 20 minutes, and the results are summarized in Figure 20. There was no significant difference between the control (H_2O_2) and the group treated with L-NMMA. This result suggests that NO is not involved in relaxation induced by H_2O_2.

5.2.3.4 Effects of H_2O_2 in the Presence of Methylene Blue

To determine the role of cGMP in H_2O_2-induced relaxation, methylene blue was used. The effects of H_2O_2 in the absence and presence of methylene blue (10^{-5} M) on ileum preparations are summarized in Figure 20. Pretreatment of tissues with methylene blue did not reduce the relaxant effect of H_2O_2, indicating no role for cGMP in the H_2O_2-induced relaxation.

5.2.3.5 Effects of Pyrilamine on H_2O_2-Induced Contraction of Ileum

Pyrilamine , a histamine receptor antagonist, was used to establish whether the H_2O_2-induced contraction of ileum is mediated through histamine. The effects of 2X

concentration of H_2O_2 in the absence and presence of pyrilamine (10^{-5} M) are summarized in Figure 21, and the results of 1X and 4X concentrations of H_2O_2 summarized in the Appendix A (Figure 9). There was no significant difference between control and groups treated with pyrilamine, indicating no involvement of histamine in the H_2O_2-induced contraction.

5.2.3.6 Effects of H_2O_2 in the presence of Atropine

Atropine, an antimuscarinic drug, was used to investigate the possible role of acetylcholine in H_2O_2-induced contractile response. The effects of 2X concentration of H_2O_2 in the absence and presence of atropine (10^{-6} M) for 20 minutes are shown in Figure 21. The results of 1X and 4X concentrations of H_2O_2 summarized in the Appendix (Figure 9). Atropine did not reduce the contractile effects of H_2O_2 on ileum preparations.

5.3 Effects of Oxygen Radicals Generated by Dihydroxy Fumaric Acid (DHF) plus Ferric Chloride ($FeCl_3$) and Adenosine Diphosphate (ADP) on Ileum Preparations

Effects of four concentrations of dihydroxy fumaric acid, adenosine diphosphate and ferric chloride on the basal tension of ileum preparation were observed for 20 minutes. The results are summarized in Figure 22. As mentioned earlier, combination of DHF, ADP and $FeCl_3$ will be

represented by the abbreviation DHF. All concentrations of DHF (1X, 2X, 4X, and 8X) produced significant contractions which were maximal at 1 minute after addition of DHF; thereafter, the tension decreased significantly. This contraction was concentration-dependent. Thus, 1X, 2X, 4X and 8X concentrations of DHF produced maximum contractions of 31.2%, 35.8%, 86.5%, and 117.8%, respectively. Significant differences between the groups were observed at times 1, 3, and 5 minutes.

5.3.1 Oxygen Metabolites Generated by DHF/$FeCl_3$-ADP System

The combination of dihydroxy fumaric acid, ferric chloride and adenosine diphosphate generates spectrum of oxidants ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$) outlines as below:

$$DHF + O_2 \longrightarrow [DHF]^{\cdot} + O_2^{\cdot -} \quad [5.1]$$

$$2H^+ + O_2^{\cdot -} + DHF \longrightarrow [DHF]^{\cdot} + H_2O_2 \quad [5.2]$$

$$O_2^{\cdot -} + Fe^{3+}\text{-ADP} \longrightarrow O_2 + Fe^{2+}\text{-ADP} \quad [5.3]$$

$$Fe^{2+}\text{-ADP} + H_2O_2 \longrightarrow Fe^{3+}\text{-ADP} + {}^{\cdot}OH + OH \quad [5.4]$$

Also, the interaction of $O_2^{\cdot -}$ with H_2O_2 via the non-enzymatic dismutation reaction results in $^1O_2$ formation:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad [5.5]$$

$$H_2O_2 + O_2^{\cdot -} \longrightarrow {}^{\cdot}OH + OH^- + {}^1O_2 \quad [5.6]$$

Therefore, in the following sections the effects of DHF in the

absence and presence of various antioxidants will be evaluated to determine which free radicals ($O_2^{\cdot -}$, H_2O_2, $\cdot OH$, or $^1O_2$) may be involved in the contraction of ileum preparations.

5.3.1.1 Effect of DHF/$FeCl_3$-ADP in the Presence of Superoxide Dismutase

The antioxidant superoxide dismutase was used to determine whether or not the contractile response induced by DHF is mediated through $O_2^{\cdot -}$. All tissues were pretreated with SOD 2-3 minutes prior to addition of DHF. The effects of 2X concentration of DHF in the absence and presence of SOD (100 U/ml) for 20 minutes are summarized in Figure 23, and the results of 4X and 8X concentrations of DHF summarized in the Appendix (Figure 10). Pretreatment with SOD did not reduce the contraction induced by DHF, indicating superoxide anion is not involved in DHF-induced contraction of ileum.

5.3.1.2 Effect of DHF/$FeCl_3$-ADP in the Presence of Catalase

Catalase, an H_2O_2 scavenger, was used to establish if DHF effects on ileum are mediated by H_2O_2. Figure 23 summarizes the effects of 2X concentration of DHF in the absence and presence of catalase (500 U/ml). The results of 4X and 8X concentrations of DHF are summarized in the Appendix (Figure 10). There were no significant differences between control (DHF) and groups treated with catalase. These results suggest that H_2O_2 is not involved in DHF-induced contraction of ileum.

Because catalase scavenges H_2O_2 and SOD scavenges $O_2^{\cdot -}$, both SOD + catalase were used in another experiment to eliminate the role of both H_2O_2 and $O_2^{\cdot -}$. Figure 23 summarizes the effects of both SOD + catalase on DHF-induced contraction. The results of 4X and 8X concentrations of DHF summarized in the Appendix (Figure 10). In the presence of SOD and catalase, the time frame of peak tension was similar to controls (DHF); however, there were no significant differences between groups. The overall results of treatment with SOD and catalase suggest that neither H_2O_2 nor $O_2^{\cdot -}$ are responsible for the contraction induced by DHF on ileum preparation.

5.3.1.3 Effect of DHF/$FeCl_3$-ADP in the Presence of Mannitol

Mannitol, an ·OH scavenger, was used to determine if hydroxyl radical plays a role in DHF-induced contraction. The effects of 2X concentration of DHF on ileum in the absence and presence of increasing concentrations of mannitol are summarized in Figure 24. The results of 4X and 8X concentrations of DHF summarized in the Appendixs Figures 11 and 12, respectively. Mannitol at two concentrations (40 and 80 mM) reduced the contraction significantly, and the effects were concentration dependent. DHF (2X) produced a maximum contraction of 35.8%; however, in the presence of 40 and 80 mM mannitol the contraction was reduced to 13.1% and 4.2% of maximum, respectively. These results support an involvement

of ·OH in DHF-induced contraction.

5.3.1.4 Effect of DHF/$FeCl_3$-ADP in the Presence of Dimethylthiourea

Dimethylthiourea , another ·OH scavenger, was used to further support ·OH involvement in the DHF-induced contraction of ileum. The effects of 2X concentration of DHF in the absence and presence of increasing concentrations of DMTU are summarized in Figure 25. The results of 4X and 8X concentrations of DHF are summarized in the Appendix, Figures 11 and 12, respectively. As mentioned earlier, the maximum contraction induced by 2X concentration of DHF was 35.8%. DMTU (36.3 μg/ml) produced a significant reduction in contraction only at 3 and 5 minutes; however, reduction of contraction with higher dose of DMTU (72.6 μg/ml) was observed at all time points. These results further support the role of ·OH in DHF-induced contraction.

5.3.1.5 Effect of DHF/$FeCl_3$-ADP in the Presence of Histidine

One of the nonenzymatic sources of singlet oxygen production has been reported to be the spontaneous dismutation of superoxide anion; therefore, it is possible that addition of DHF could produce $^1O_2$. Histidine, a singlet oxygen scavenger, was used in this study to determine the possible role of singlet oxygen in the DHF-induced contraction of ileum. Figure 26 summarizes the effects of 2X

concentration of DHF in the absence and presence of histidine. Histidine (100 mM) reduced the contraction induced by DHF after 5 minutes, and there was a significant difference between control and treated group at times 10, 15 and 20 minutes. Pretreatment with histidine reduced the contraction by 46.35%. These results suggest that $^1O_2$ may also play a role in DHF-induced contraction.

5.3.2 Mechanisms of DHF/$FeCl_3$-ADP-Induced Contraction

5.3.2.1 Effect of DHF/$FeCl_3$-ADP in the Presence of Indomethacin

Indomethacin, the cyclooxygenase inhibitor, was used to determine whether or not DHF-induced contractions of the ileum preparation are mediated through arachidonic acid metabolites. The results of the effects of 2X concentration of DHF in the absence and presence of increasing concentrations of indomethacin are summarized in Figure 27. The results of 4X and 8X concentrations of DHF summarized in the Appendix (Figure 13). Indomethacin inhibited the contraction induced by DHF significantly, in a concentration-dependent manner. Indomethacin significantly reduced the contraction-induced by all concentrations of DHF at all time points. Pretreatment with indomethacin ($2x10^{-5}$ and $4x10^{-5}$ M) reduced the maximum contraction at 2X DHF by 21.3 and 86.4%, respectively.

5.3.2.2 Effect of DHF/$FeCl_3$-ADP in the Presence of Atropine

Effects of 2X concentration of DHF on the ileum preparation in the absence and presence of atropine are summarized in Figure 28, and the results of 4X and 8X concentrations of DHF summarized in the Appendix A, Figures 14 and 15, respectively. Atropine, an antimuscarinic drug, was used to investigate the possible role of acetylcholine in the DHF-induced contraction of ileum. Pretreatment with atropine (10^{-6} M) did not reduce the contraction-induced by DHF. This result suggests that acetylcholine is not involved in the DHF-induced contraction of ileum.

5.3.2.3 Effect of DHF/$FeCl_3$-ADP in the Presence of Pyrilamine

Pyrilamine , a histamine receptor antagonist, was used to establish whether or not the DHF-induced contraction of ileum is mediated through histamine. Therefore, the ileum strips were pretreated with two concentrations of pyrilamine 20 minutes before addition of DHF. The effects of 2X concentration of DHF in the absence and presence of pyrilamine (10^{-5} and 10^{-4} M) on ileum preparations are summarized in Figure 29, and results of 4X and 8X concentrations of DHF summarized in the Appendix, Figures 14 and 15, respectively. Pyrilamine (10^{-5} M) alone did not affect basal tone of the ileum; however, pyrilamine at concentration of 10^{-4} M produced a small contraction which was not significant and remained constant during the experiment. The

contractile effects of DHF were significantly reduced in the presence of pyrilamine, suggesting a role of histamine in DHF-induced contraction of ileum. DHF (2X) produced a maximum contraction of 35.76%. Pyrilamine, in a concentration-dependent manner, reduced the initial contraction by 62.83% (i.e., from 35.76 to 13.28%) with 10^{-5} M, and by approximately 100% with 10^{-4} M. These results suggest that histamine is partially involved in the DHF-induced contraction of ileum.

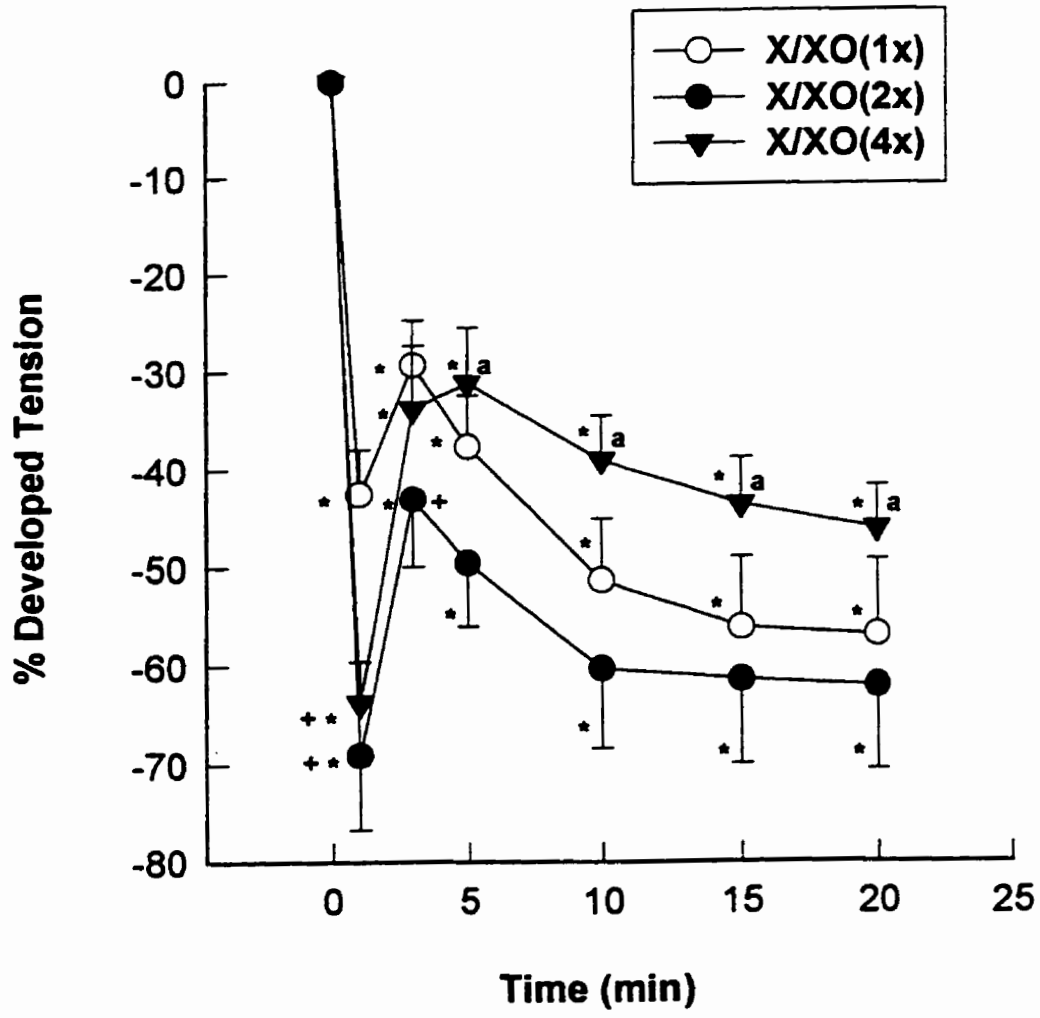

Figure 1: Effects of various concentrations of xanthine plus xanthine oxidase (1X, 2X, and 4X) on basal tone of ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, 1X vs 2X or 4X.
***a* P<0.05, 2X vs 4X.**

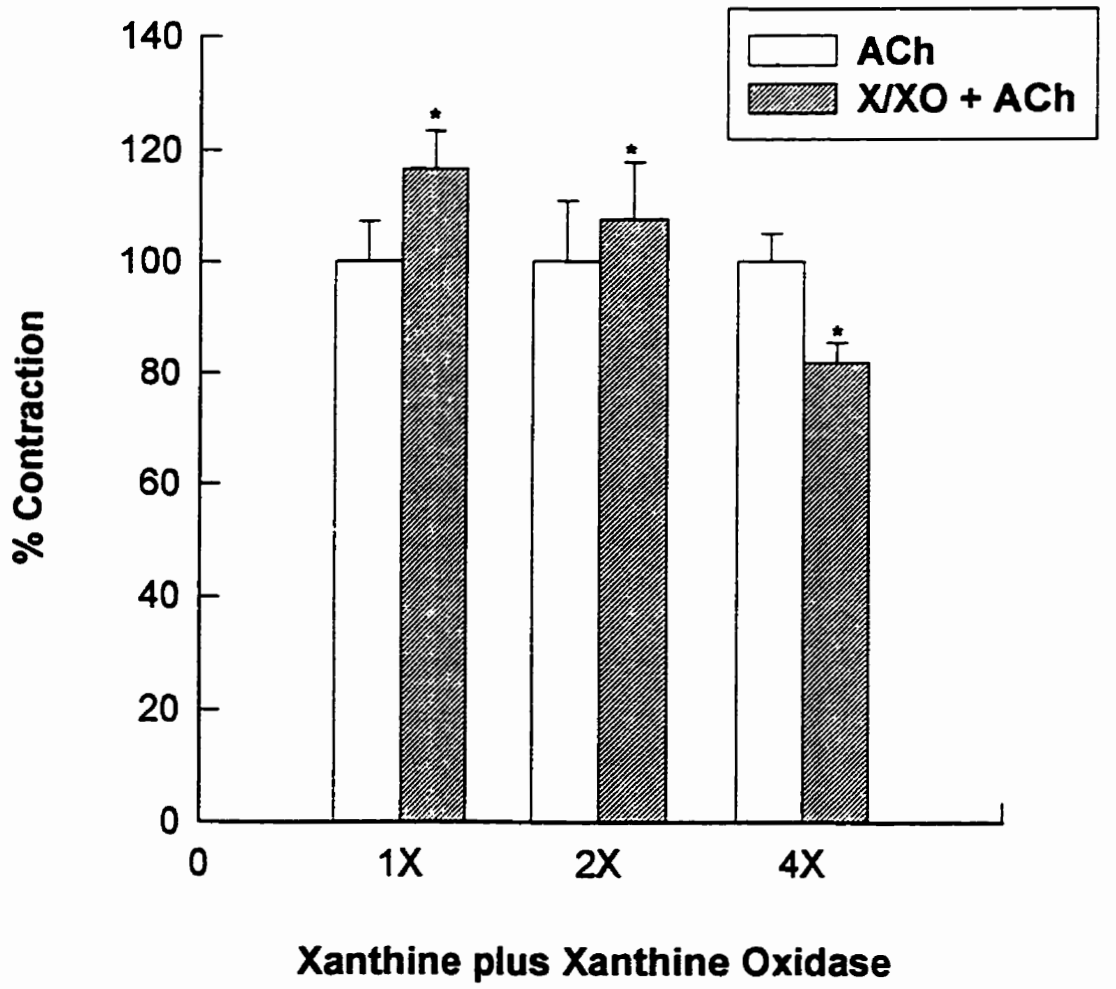

Figure 2: Effects of acetylcholine (ACh, 0.5 μg/ml) in absence or presence of three concentrations of xanthine plus xanthine oxidase (X/XO) on basal tension of ileum preparations.

Results are expressed as % change from maximal ACh response taken as 100%.
* P<0.05, comparison of values after exposure to various concentrations of X/XO with respect to values before exposure to X/XO within groups.

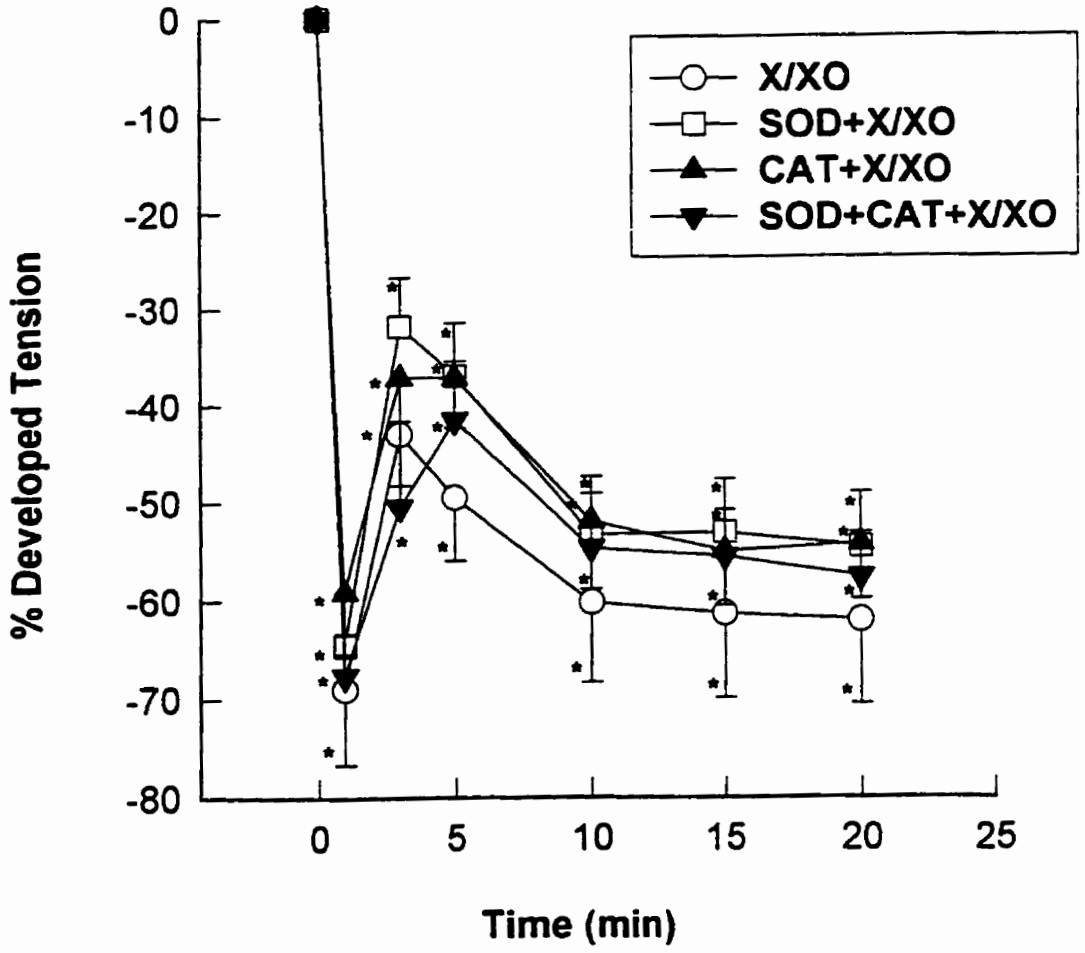

Figure 3: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of 100 U/ml superoxide dismutase (SOD), 500 U/ml catalase (CAT), and SOD (100 U/ml)+CAT(500 U/ml)on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**

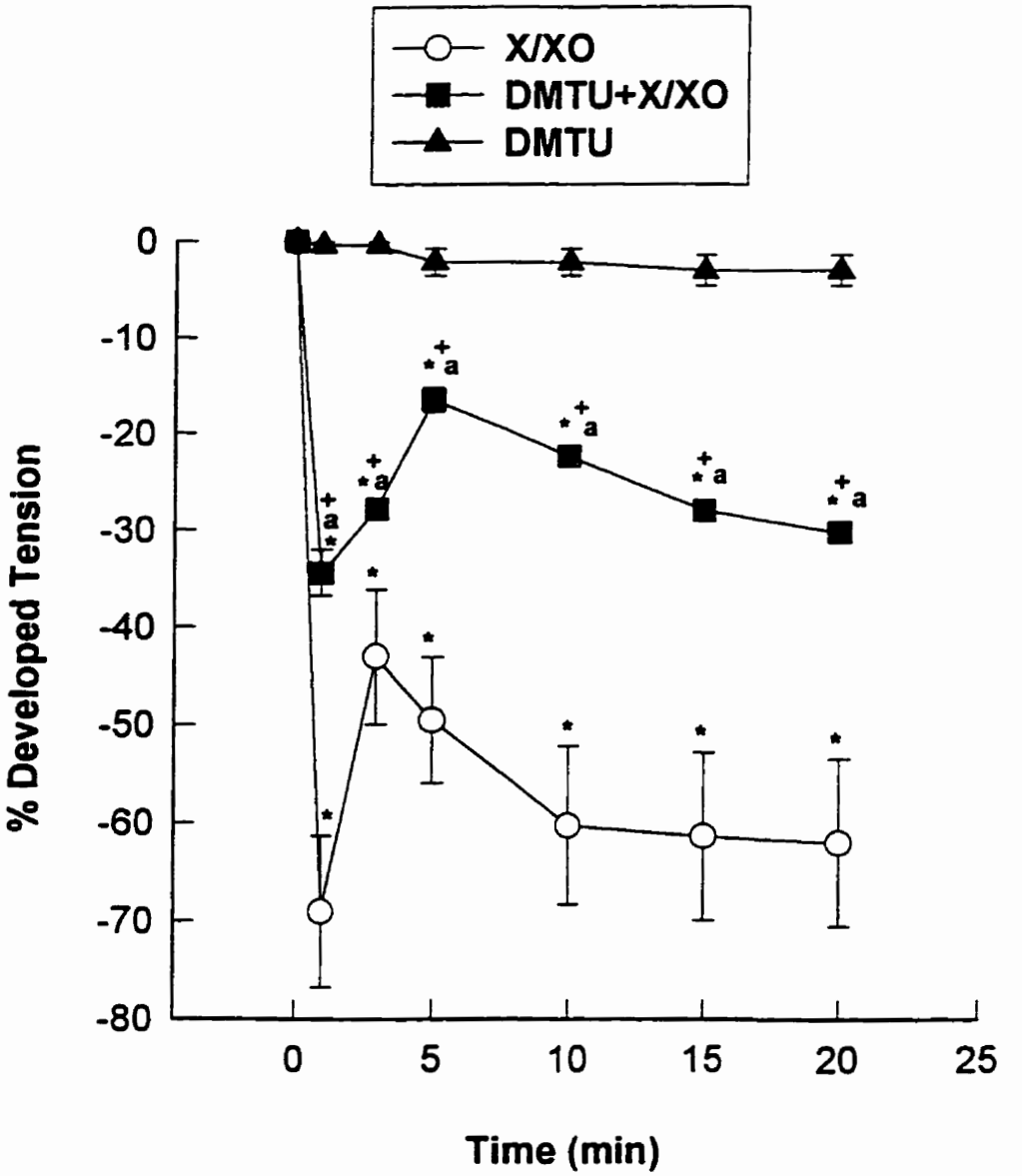

Figure 4: Effect of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, X/XO vs DMTU (1.6 mg/ml) + X/XO.
a P<0.05, DMTU vs DMTU + X/XO.

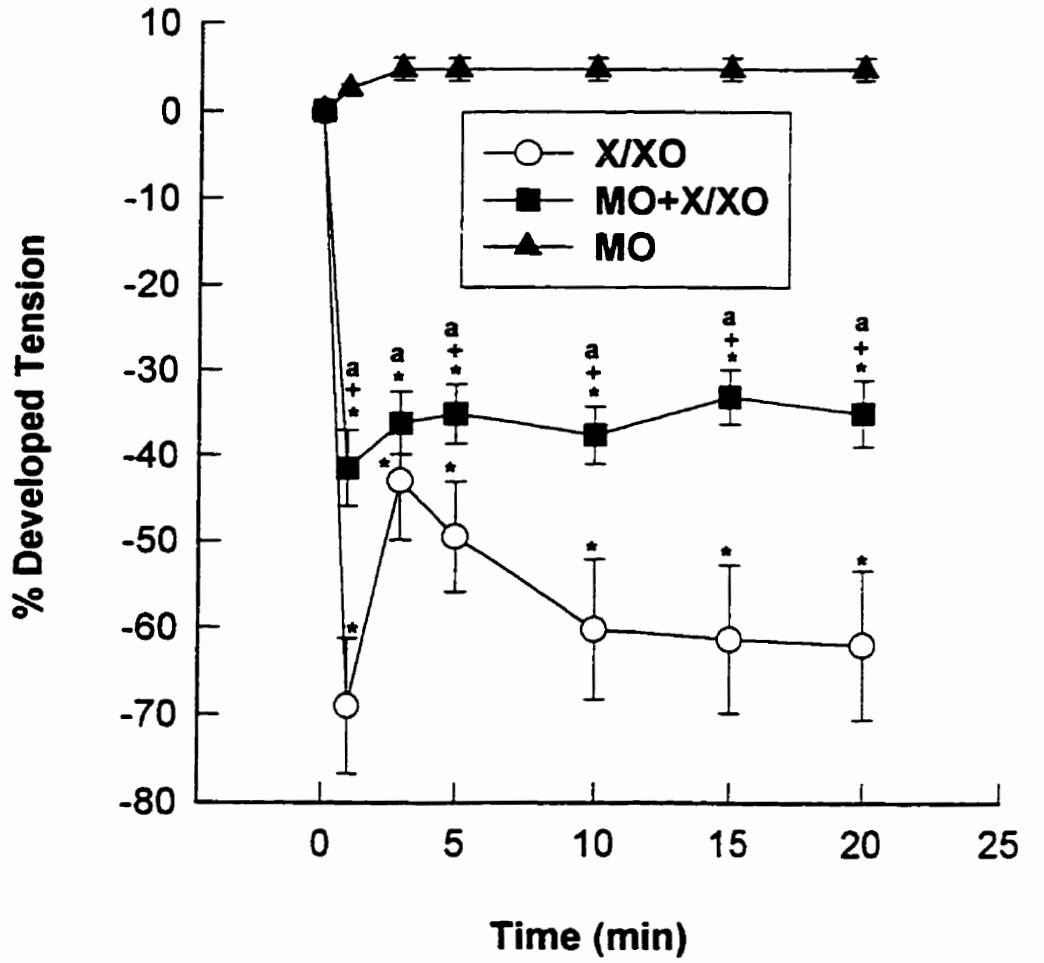

Figure 5: Effect of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of mannitol (MO) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, X/XO vs MO (80 mM) + X/XO.
a P<0.05, MO vs MO + X/XO.

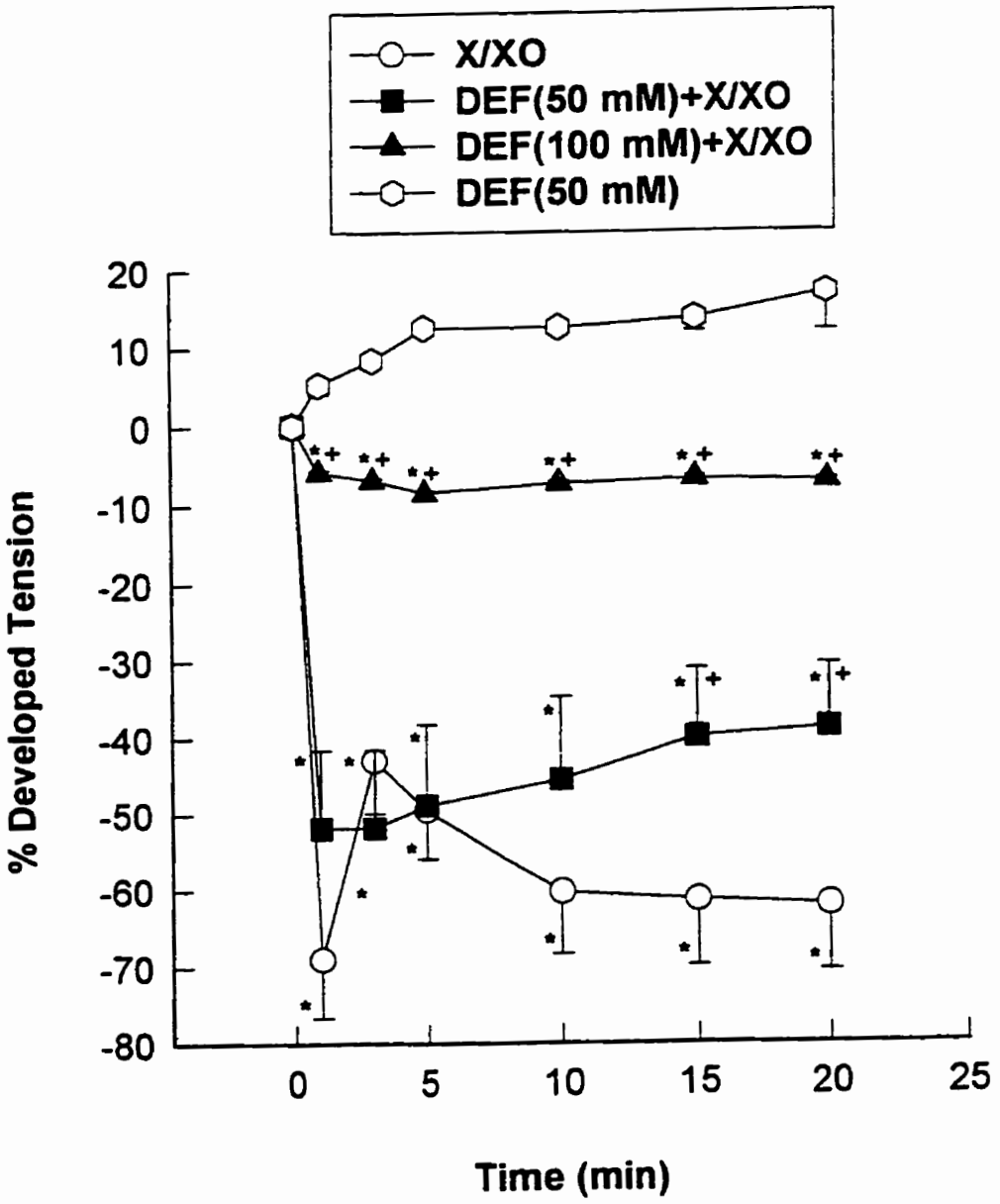

Figure 6: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of deferoxamine (DEF) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, X/XO vs DEF (50 mM) + X/XO or DEF (100 mM) + X/XO.

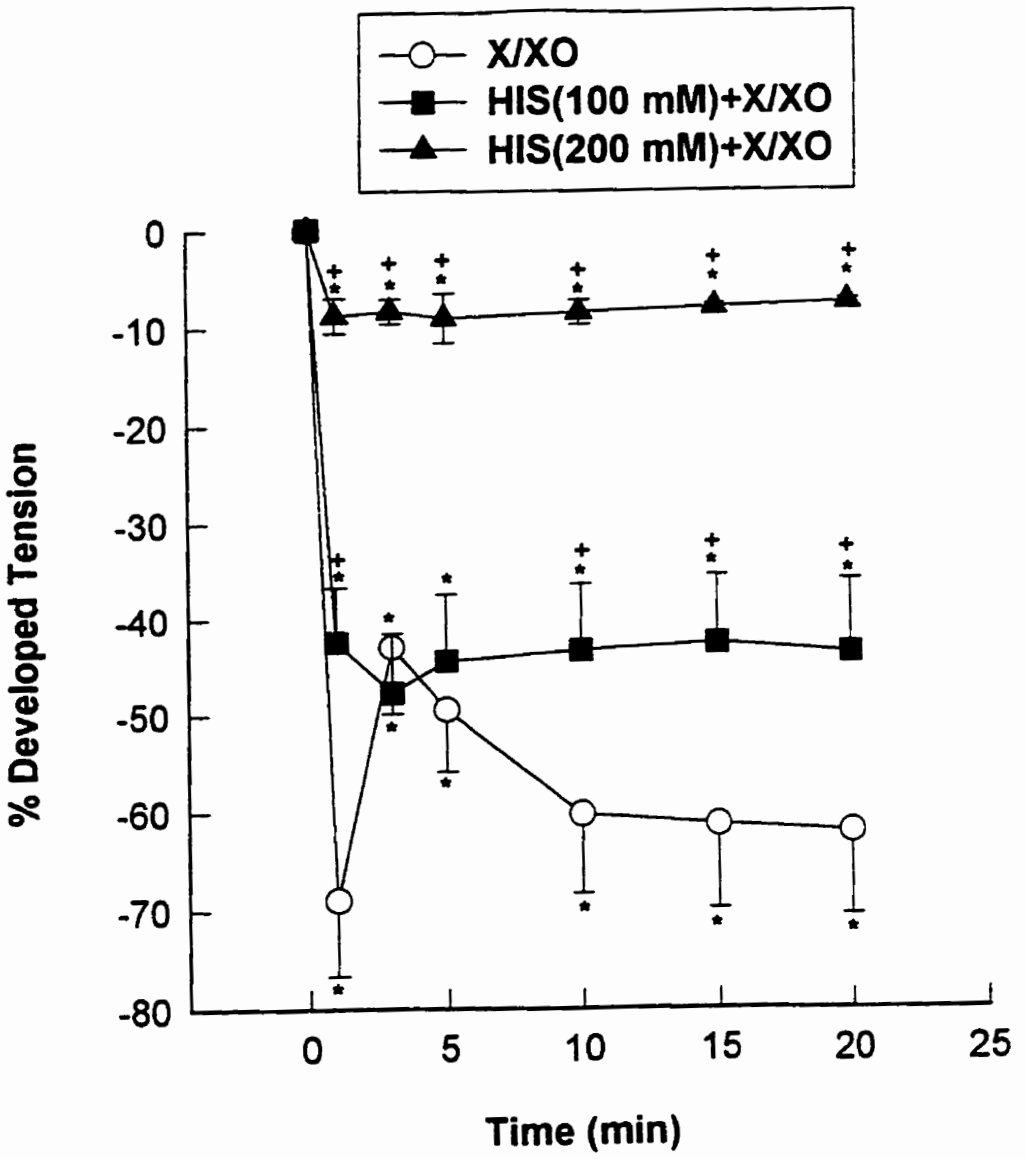

Figure 7: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of histidine (HIS) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, X/XO vs HIS (100 mM) + X/XO or HIS (200 mM) + X/XO.

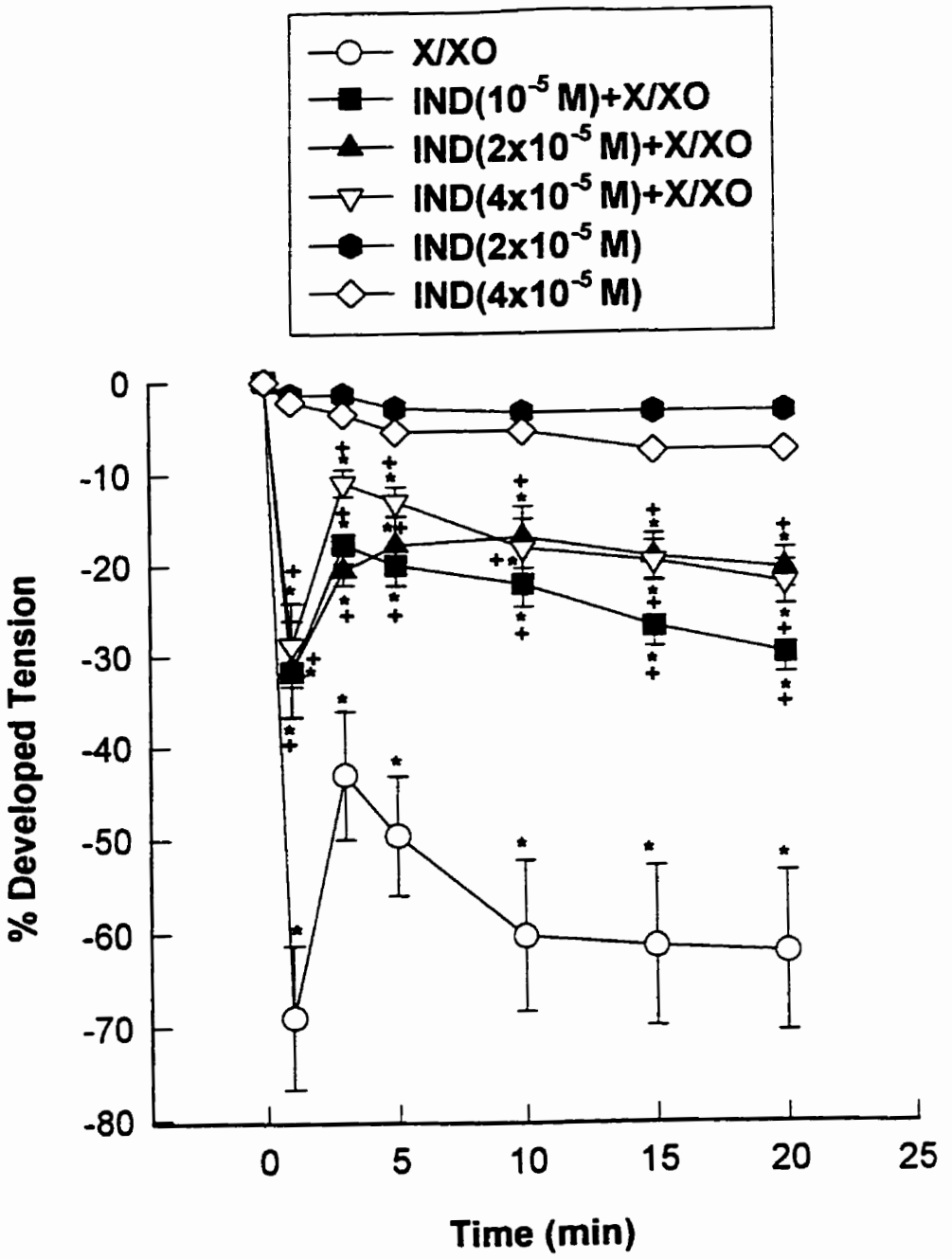

Figure 8: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of various concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, X/XO vs IND (10^{-5} M) + X/XO, or IND ($2x10^{-5}$ M) + X/XO, or IND ($4x10^{-5}$ M) + X/XO.

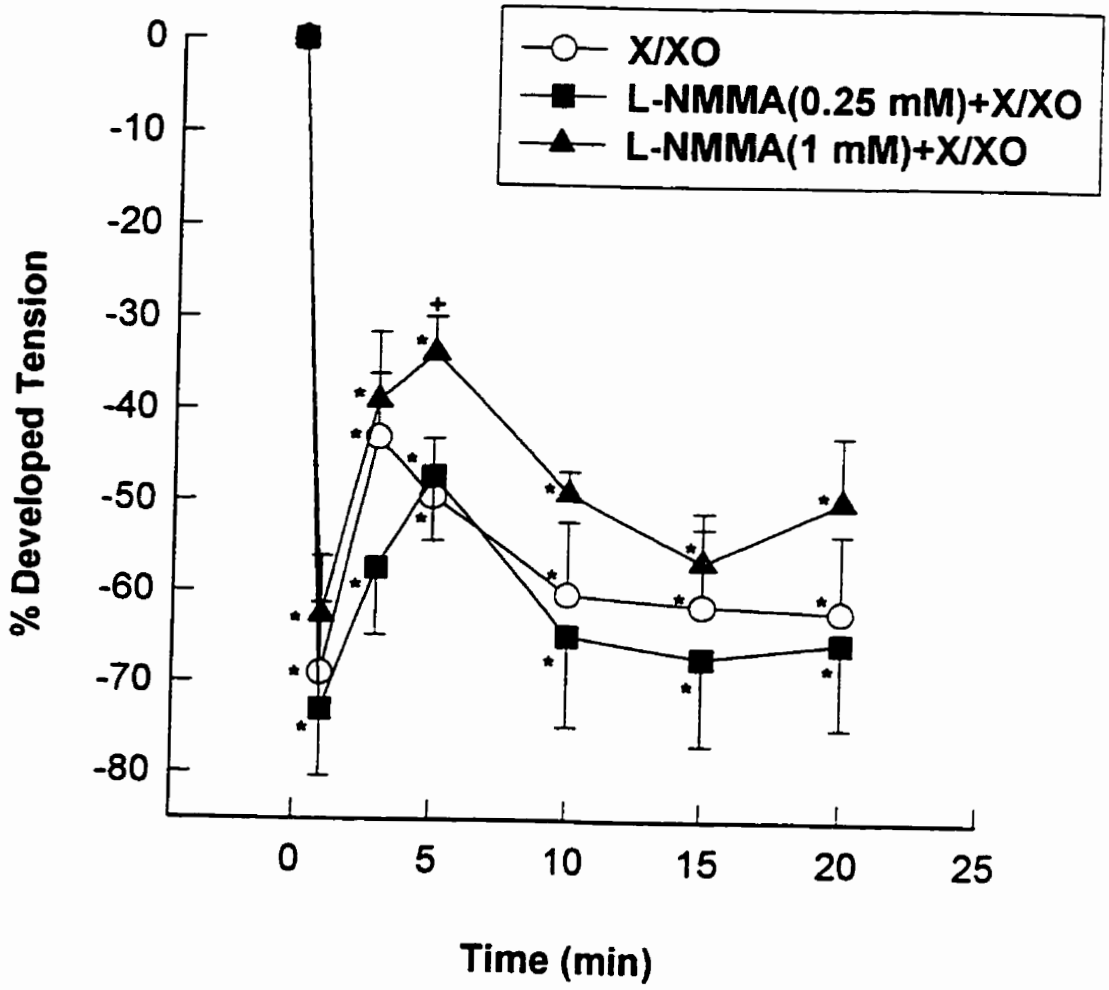

Figure 9: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of L-NMMA on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, X/XO vs L-NMMA (0.25 mM) + X/XO or L-NMMA (1 mM) + X/XO.

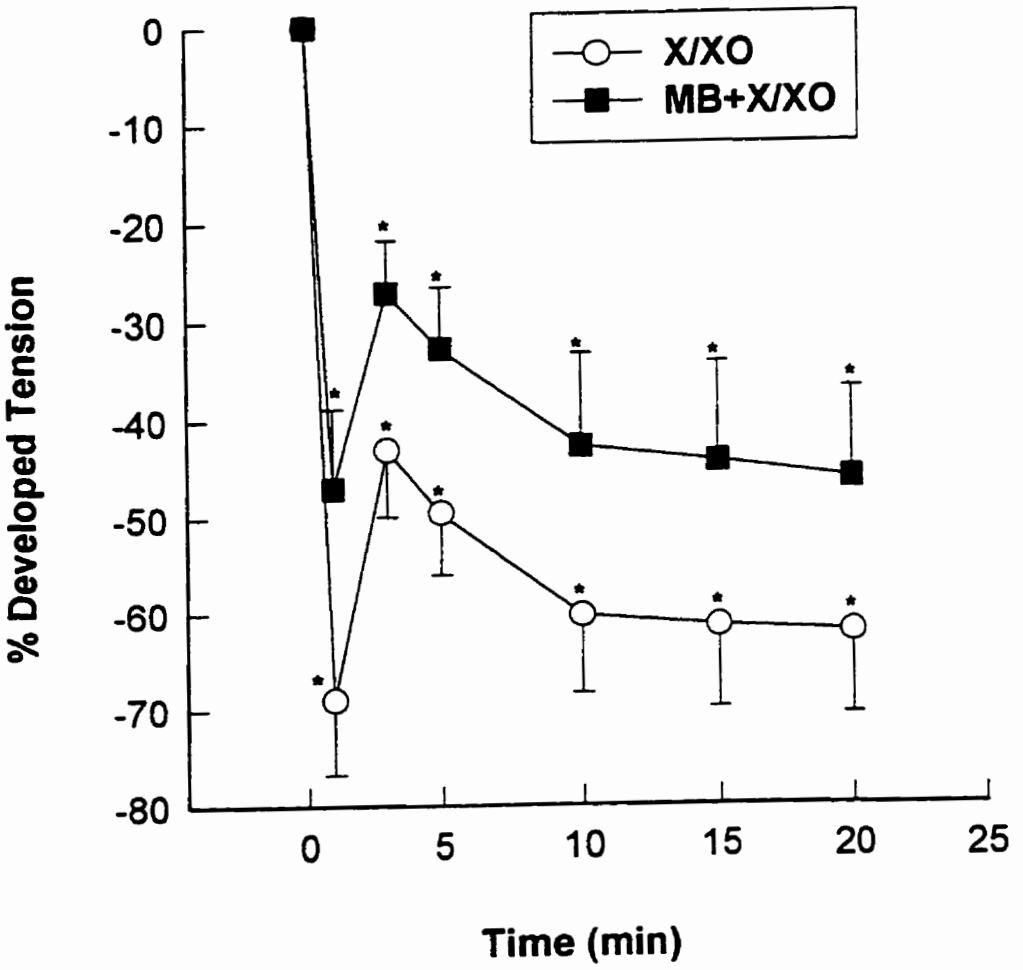

Figure 10: Effect of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of methylene blue (MB, 10^{-5} M) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups**

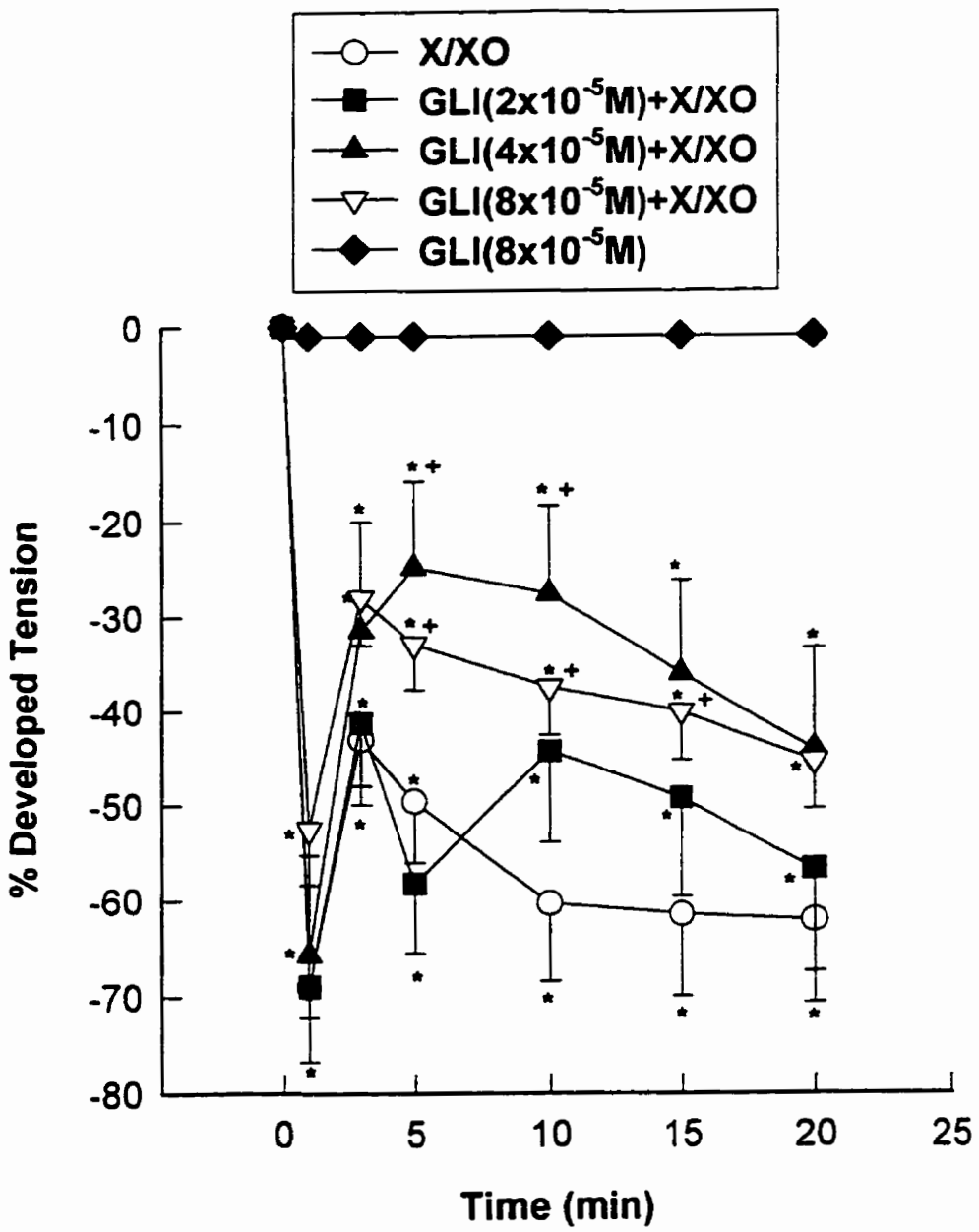

Figure 11: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of various concentrations of glibenclamide (GLI) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, X/XO vs GLI ($2x10^{-5}$ M) + X/XO, or GLI($4x10^{-5}$ M) + X/XO, or GLI($8x10^{-5}$ M) + X/XO.

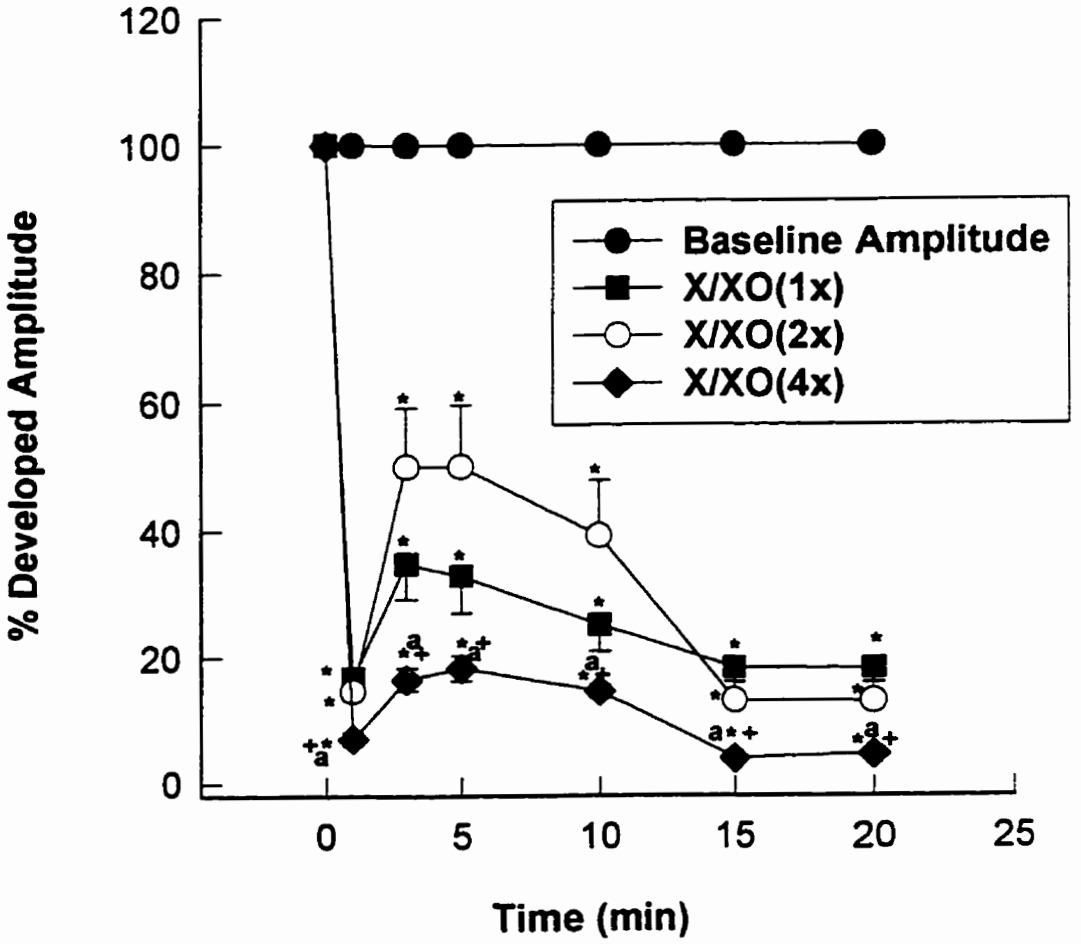

Figure 12: Effect of three concentrations of xanthine plus xanthine oxidase (X/XO) on amplitude of spontaneous activity of ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, baseline amplitude vs X/XO (1X) or X/XO (2X) or X/XO (4X).**
+ P<0.05, X/XO (1X) vs X/XO (2X) or X/XO (4X).
a P<0.05, X/XO (2X) vs X/XO (4X).

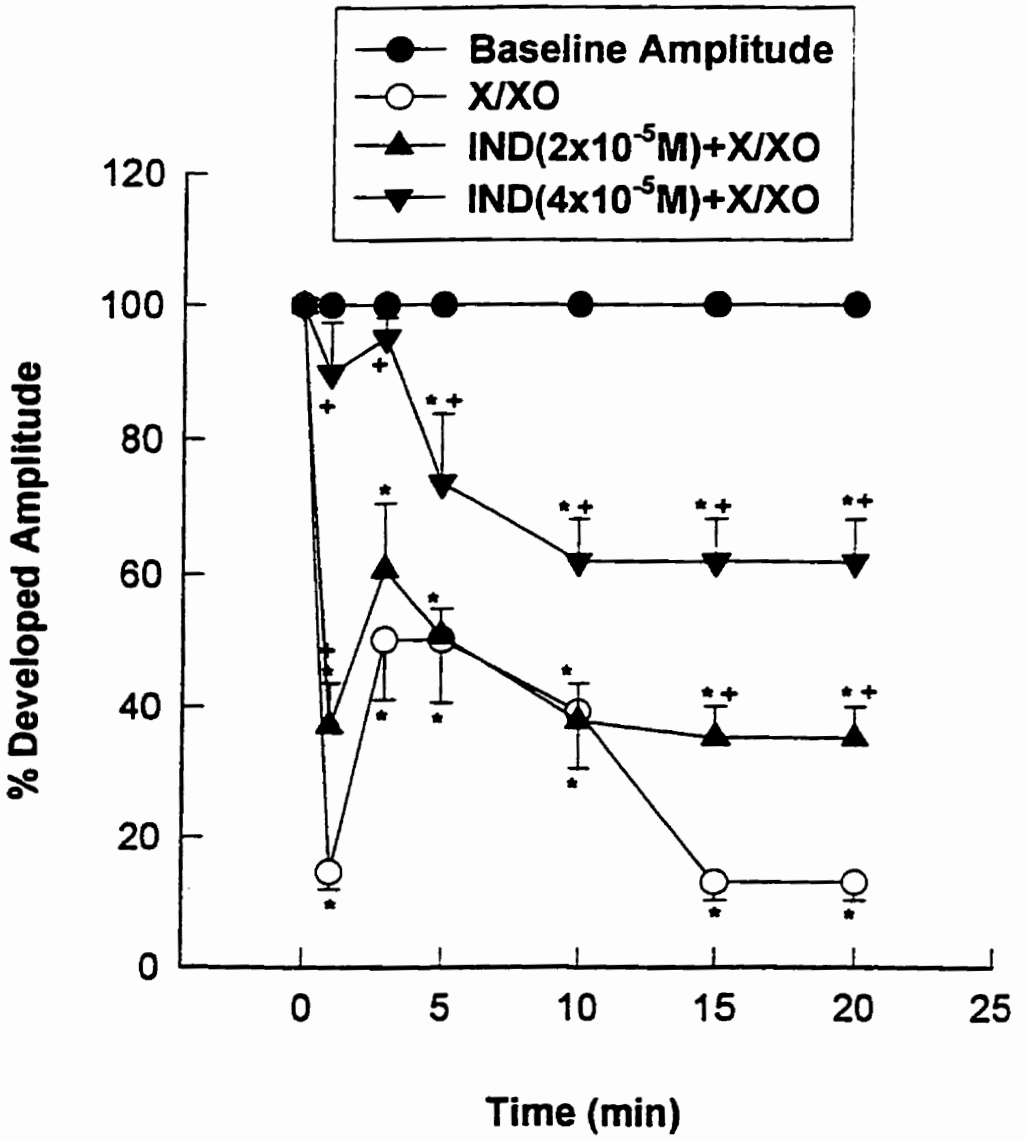

Figure 13: Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of indomethacin (IND) on amplitude of spontaneous activity of ileum strips.

Results are expressed as mean ± S.E.
* P<0.05, Baseline amplitude vs X/XO, or IND ($2x10^{-5}$ M) + X/XO, or IND ($4x10^{-5}$ M) + X/XO.
+ P<0.05, X/XO vs IND ($2x10^{-5}$ M) + X/XO or IND ($4x10^{-5}$ M) + X/XO.

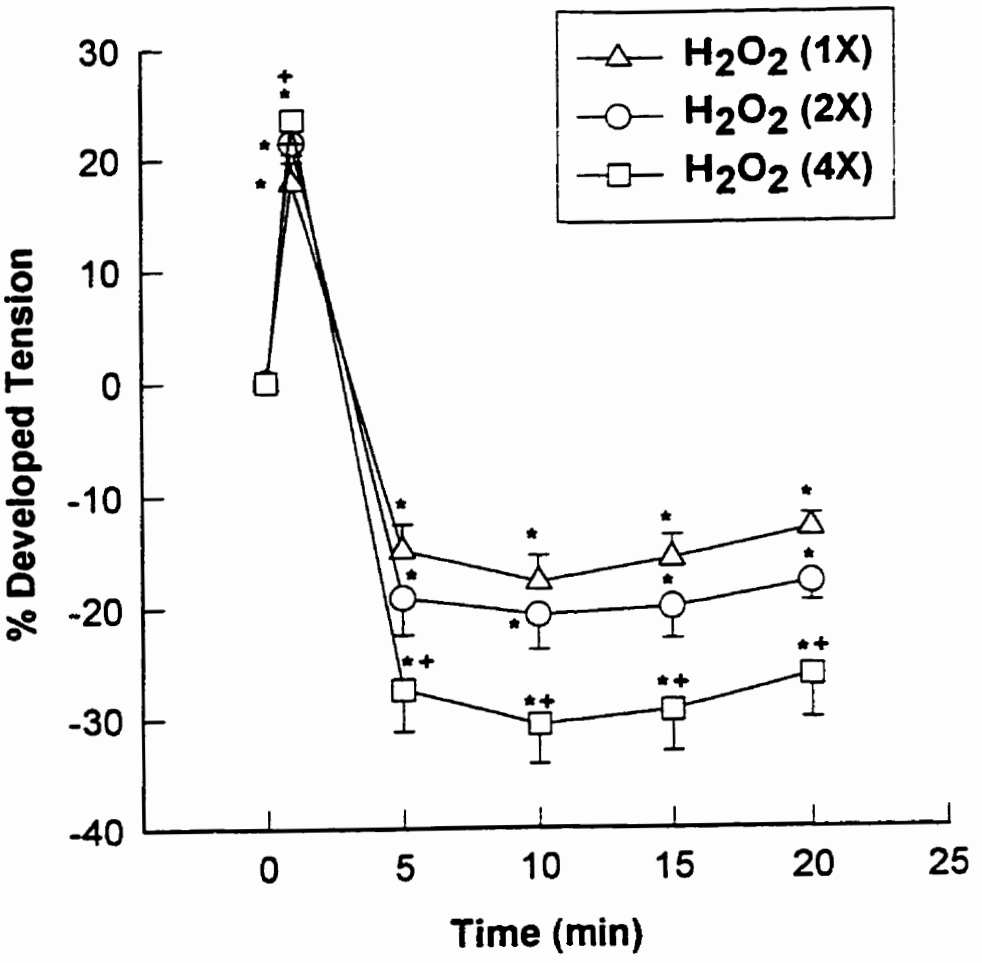

Figure 14: Effects of three concentrations of H_2O_2 on basal tone of ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" times within groups.**
\+ P<0.05, 1X vs 2X or 4X.

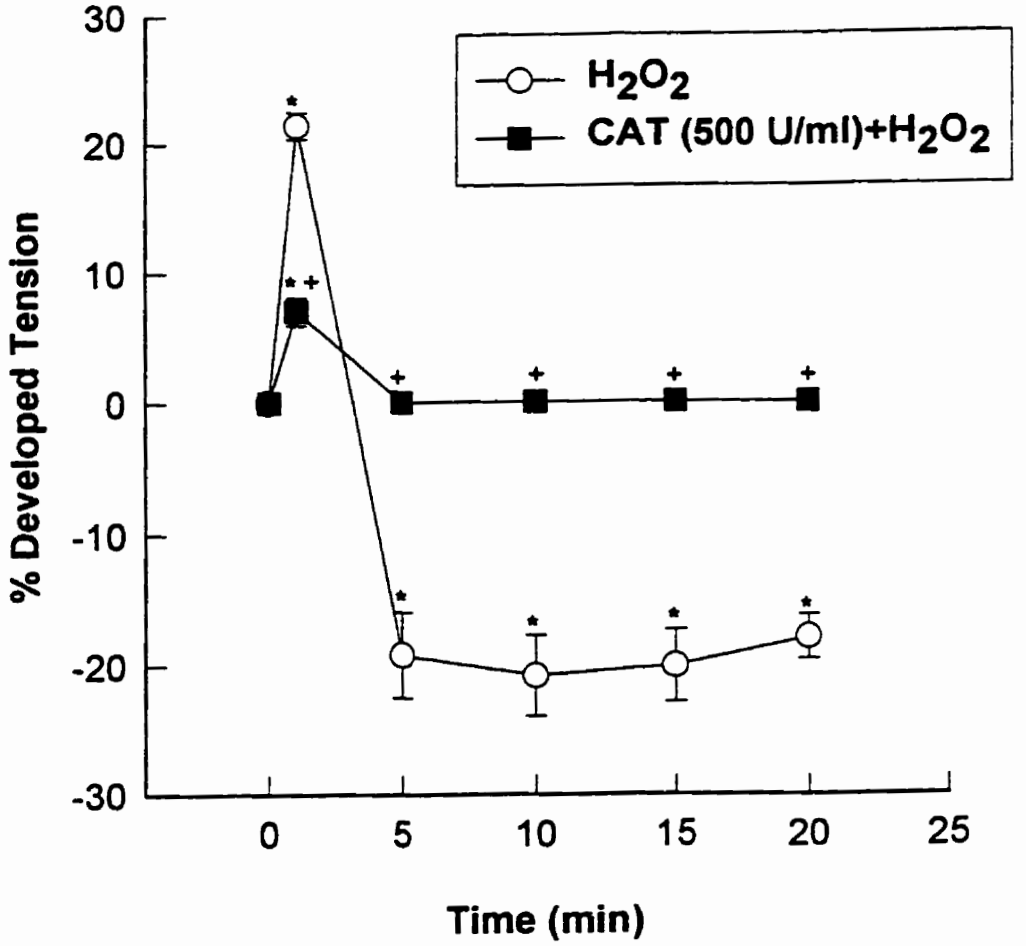

Figure 15: Effect of 2X concentration of H_2O_2 in absence or presence of catalase (CAT) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, H_2O_2 vs CAT (500 U/ml) + H_2O_2.

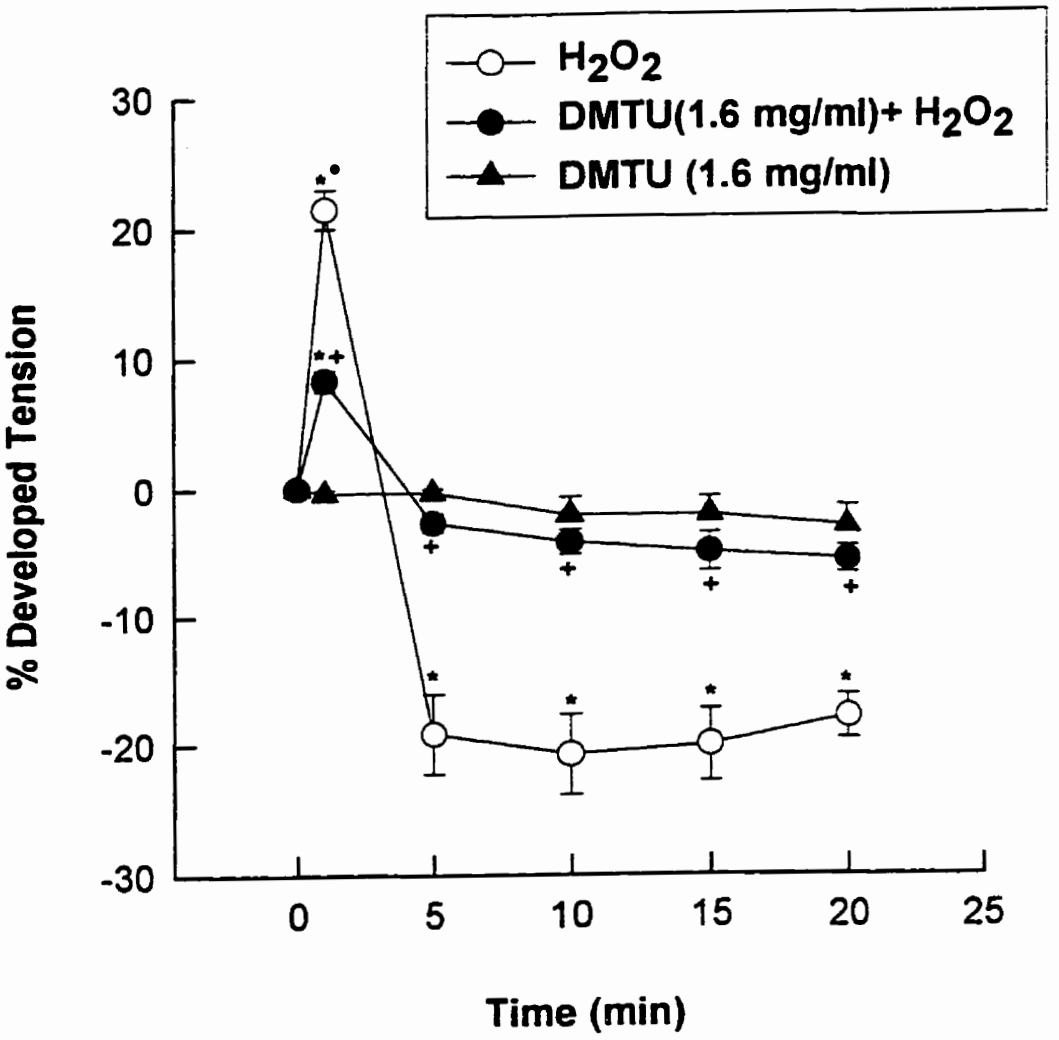

Figure 16: Effect of 2X concentration of H_2O_2 in absence or presence of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, H_2O_2 vs DMTU (1.6 mg/ml) + H_2O_2.

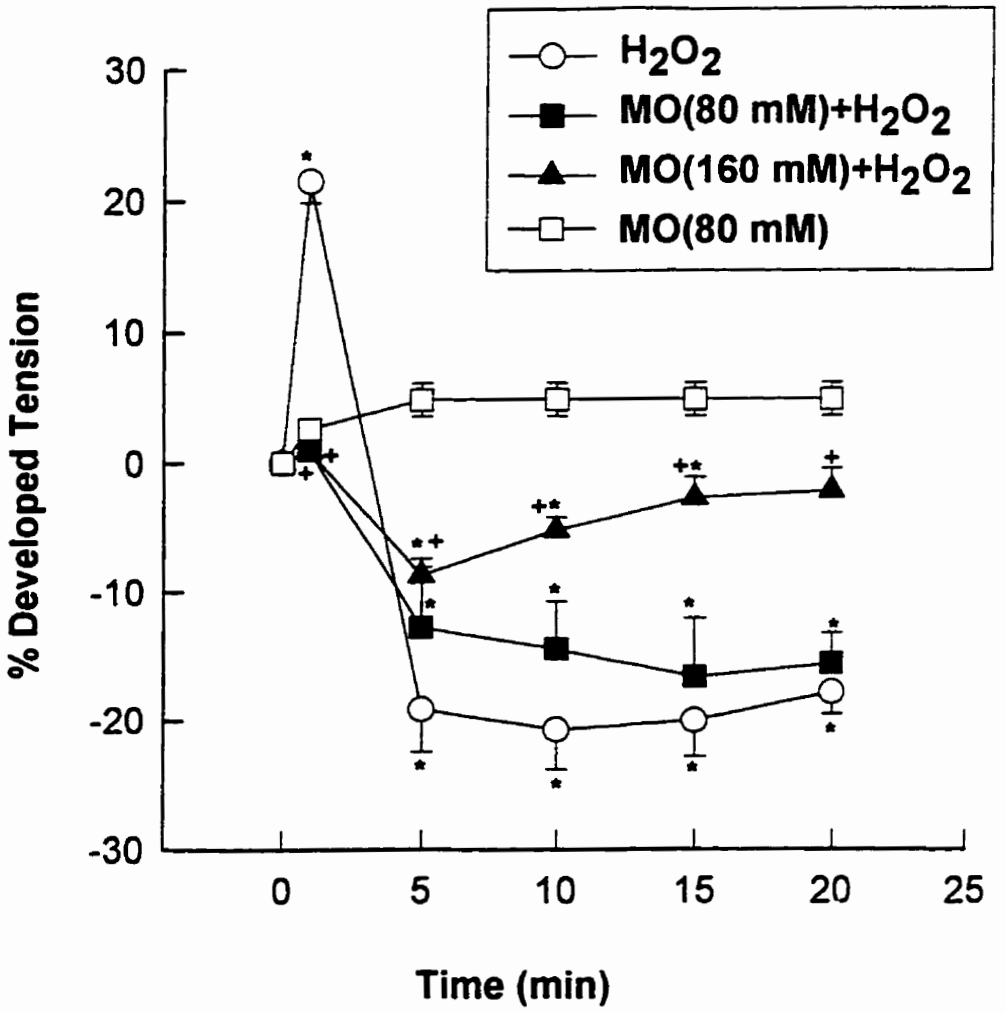

Figure 17: Effects of 2X concentration of H_2O_2 in absence or presence of two concentrations of mannitol (MO) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
\+ P<0.05, H_2O_2 vs MO (80 mM) + H_2O_2, or MO (160 mM)+ H_2O_2.

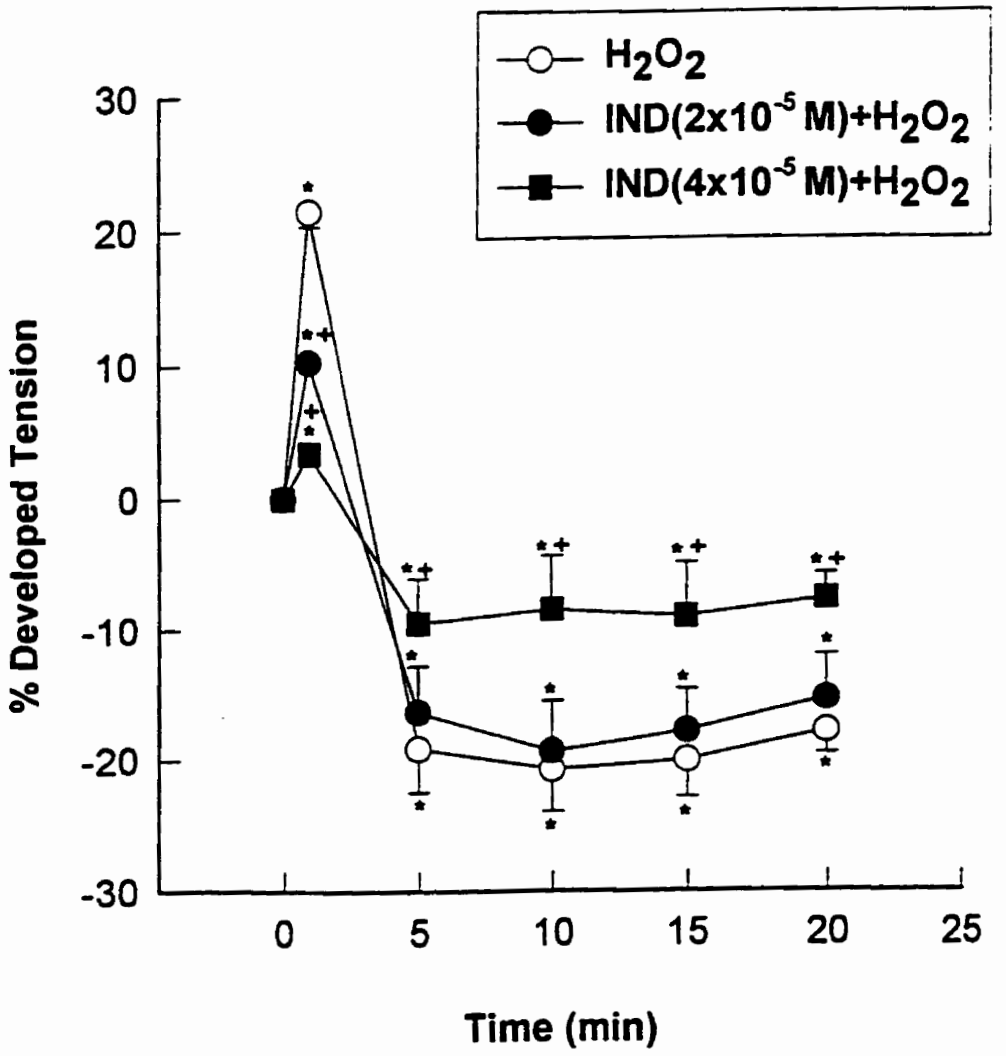

Figure 18: Effects of 2X concentration of H_2O_2 in absence or presence of various concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
*P<0.05, comparison of values at different times with respect to values at "0" time within groups
+ P<0.05, H_2O_2 vs IND($2x10^{-5}$ M) + H_2O_2, or IND($4x10^{-5}$ M) + H_2O_2.

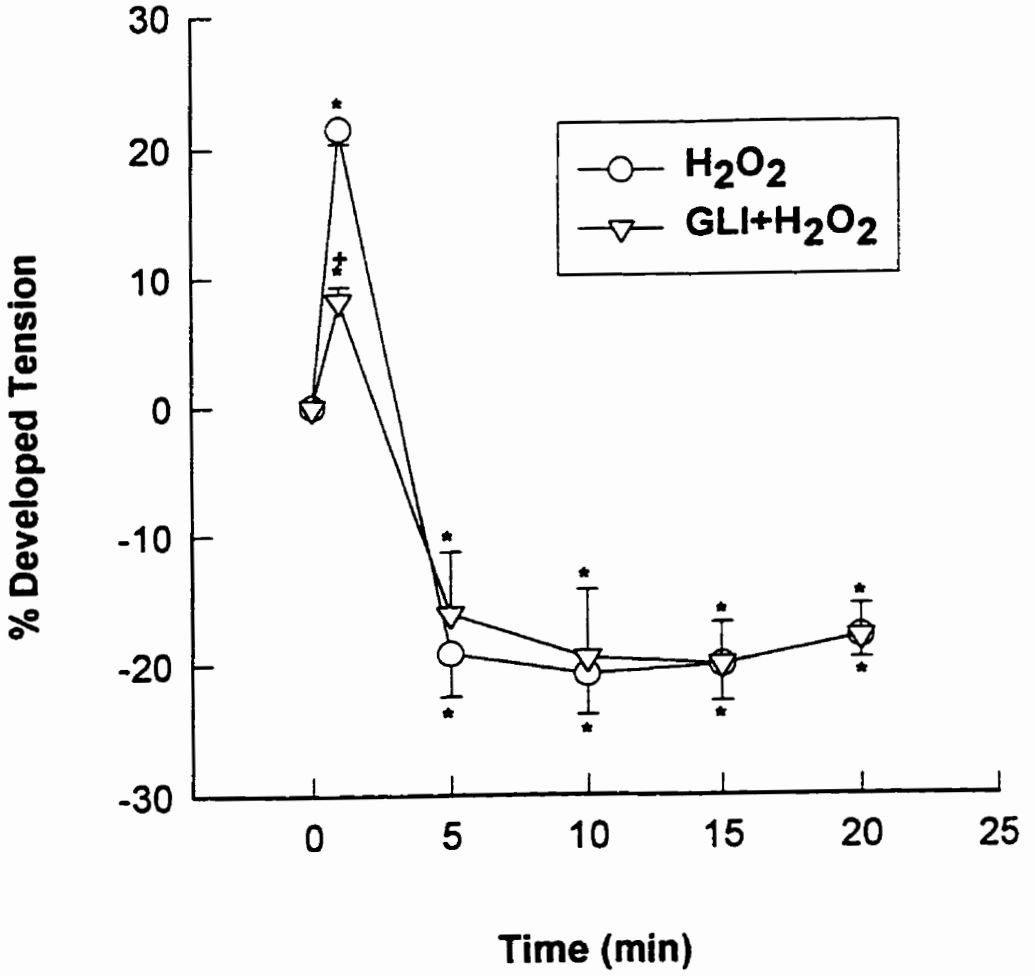

Figure 19: Effect of 2X concentration of H_2O_2 in absence or presence of glibenclamide (GLI) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
‡ P<0.05, H_2O_2 vs GLI(10^{-5} M) + H_2O_2.

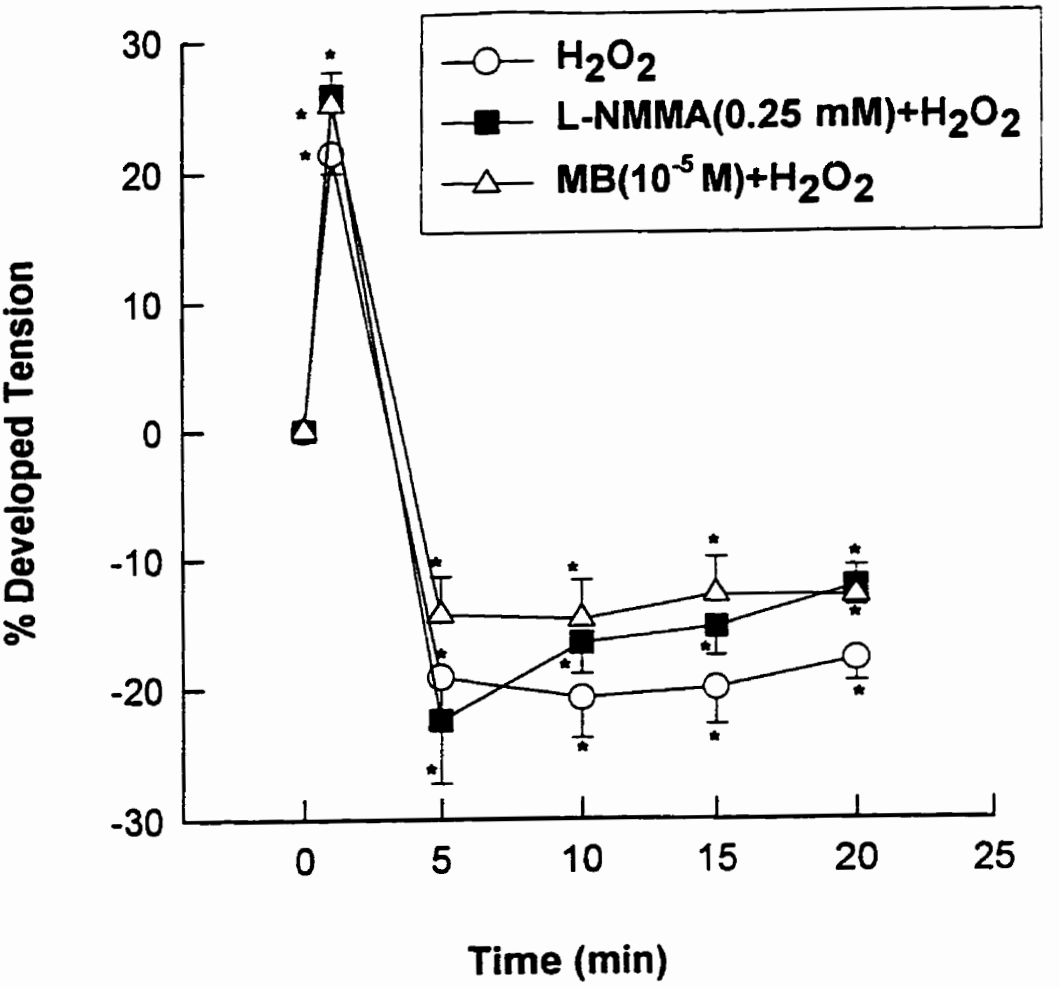

Figure 20: Effects of 2X concentration of H_2O_2 in absence or presence of L-NMMA or methylene blue (MB) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.

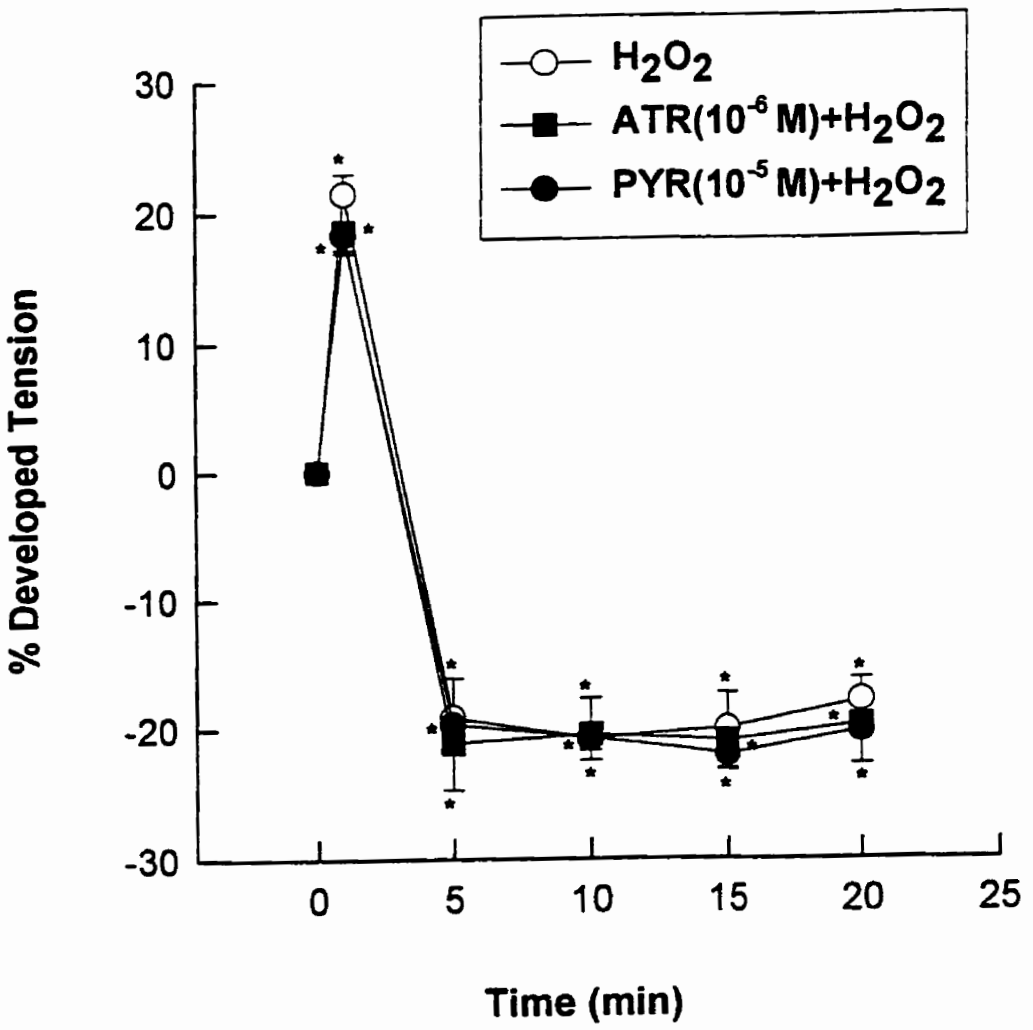

Figure 21: Effects of 2X concentration of H_2O_2 in absence or presence of atropine (ATR) or pyrilamine (PYR) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.

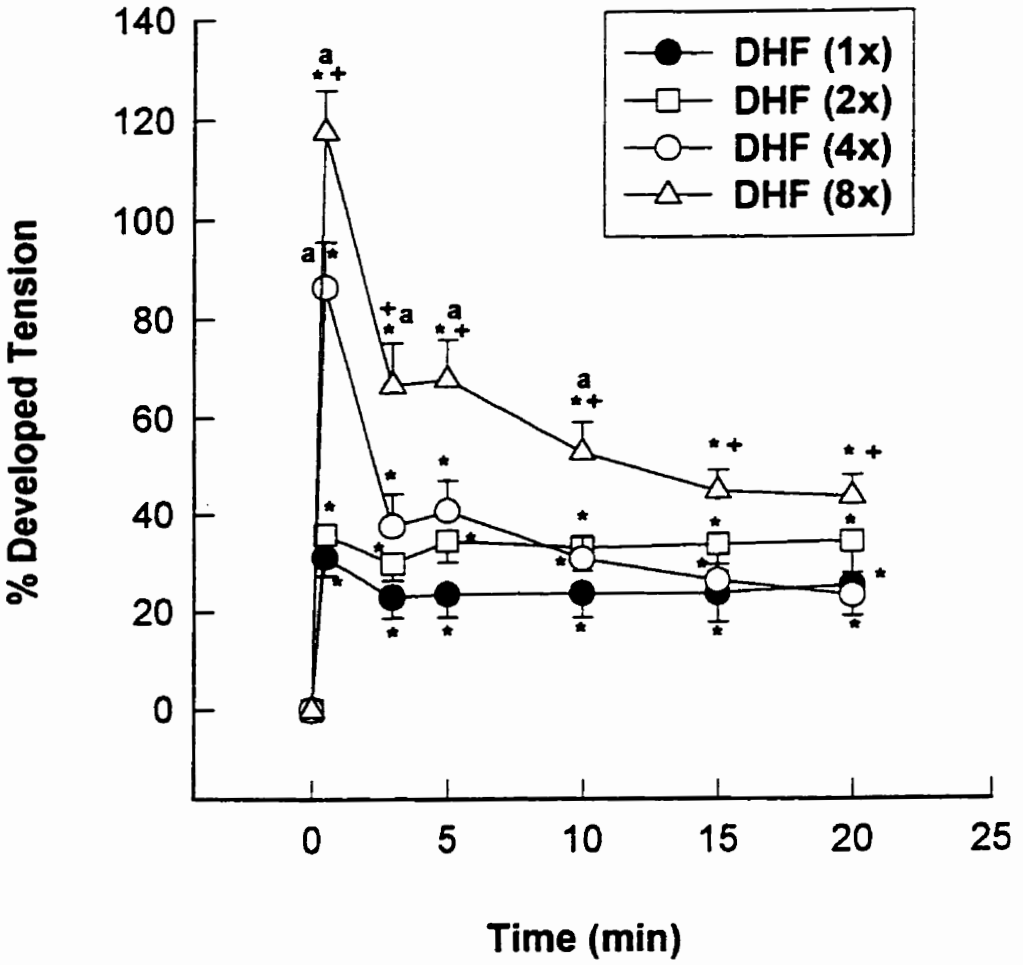

Figure 22: Effects of four concentrations (1X, 2X, 4X, and 8X) of DHF on basal tone of ileum preparations. Developed tension is expressed as percentage of basal tension.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, 1X vs 2X, or 4X, 8X.
a P<0.05, 2X vs 4X or 8X.

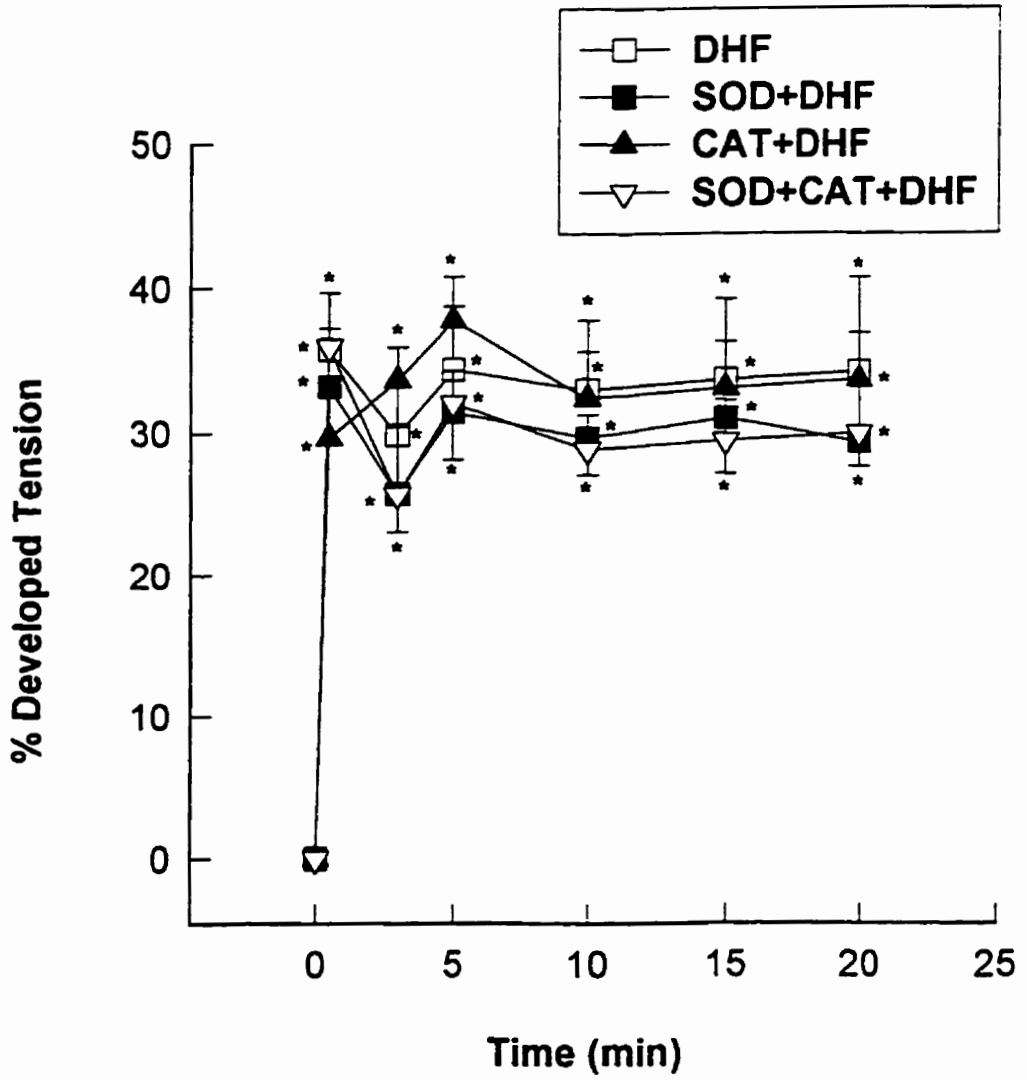

Figure 23: Effects of 2X concentration of DHF in absence or presence of superoxide dismutase (SOD, 100 U/ml), catalase (CAT, 500 U/ml), and superoxide dismutase plus catalase (SOD+CAT) on ileum preparations.

Results are expressed as mean ± S.E.
* $P<0.05$, comparison of values at different times with respect to values at "0" time within groups.

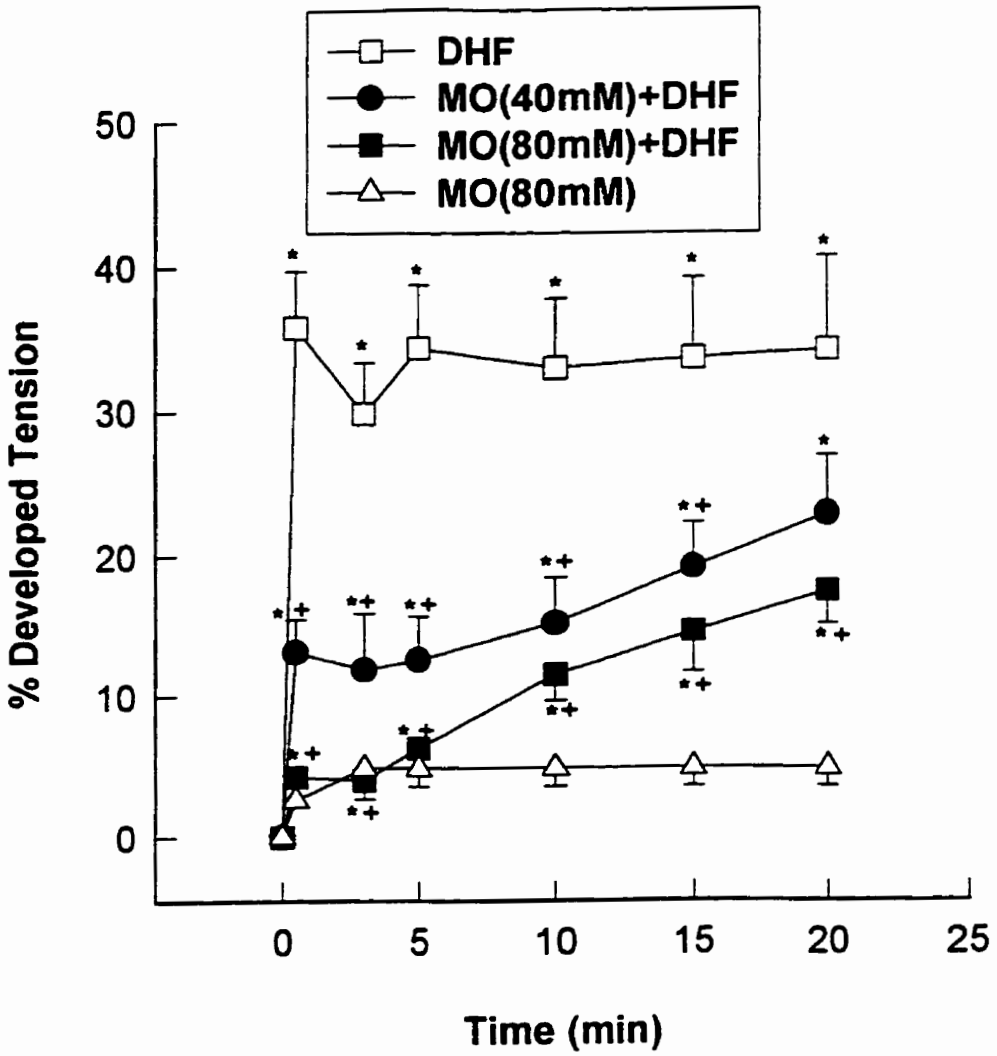

Figure 24: Effects of 2X concentration of DHF in absence or presence of two concentrations of mannitol (MO) on ileum preparations.

Results are expressed as mean ± S.E.
*P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+P<0.05, DHF vs MO(40 mM)+DHF, or MO(80 mM)+DHF.

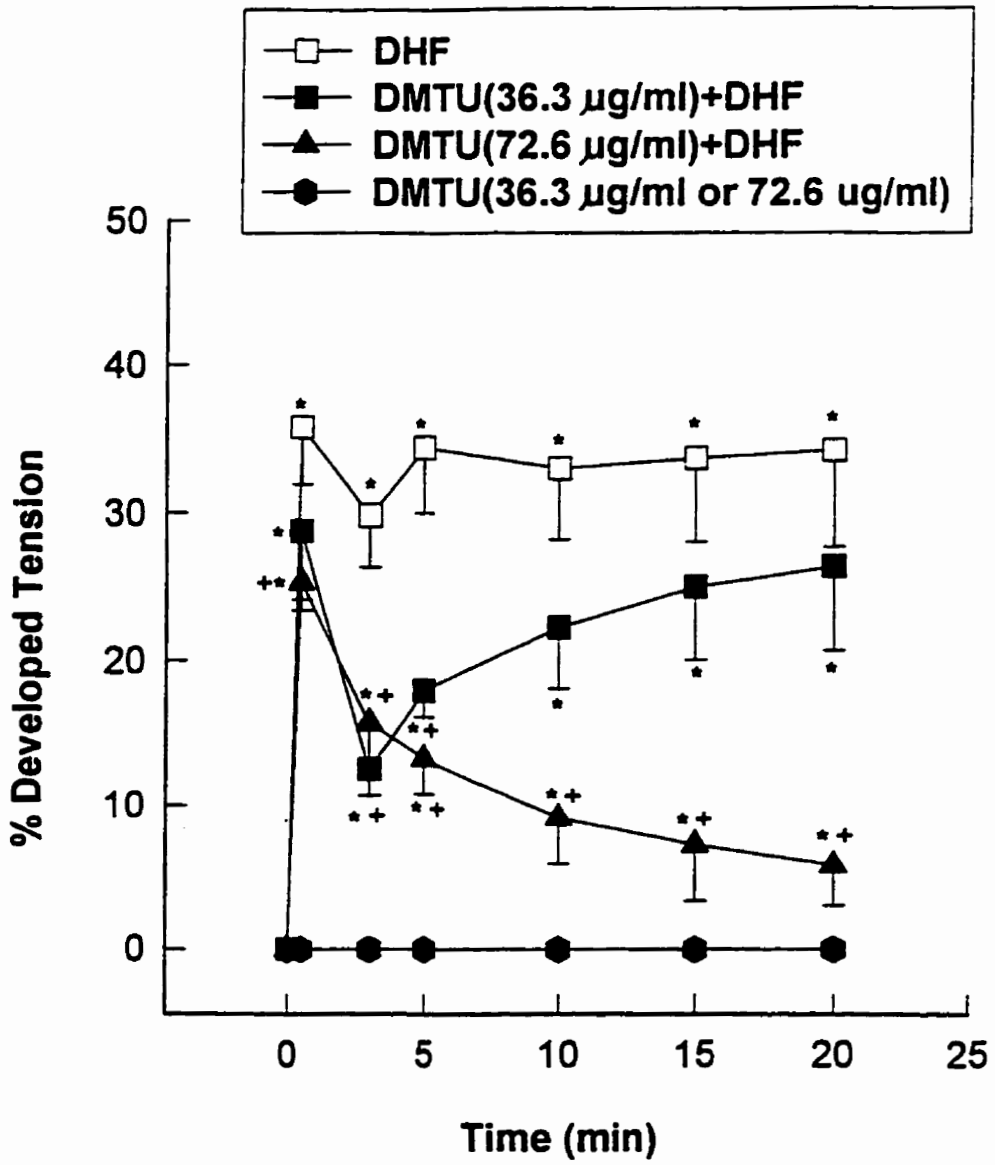

Figure 25: Effects of 2X concentration of DHF in absence or presence of two concentrations of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± S.E.
* P<0.05, comparison of values at different times with respect to values at "0" time within groups.
+ P<0.05, DHF vs DMTU(36.3 µg/ml)+DHF, or DMTU(72.6 µg/ml)+DHF.

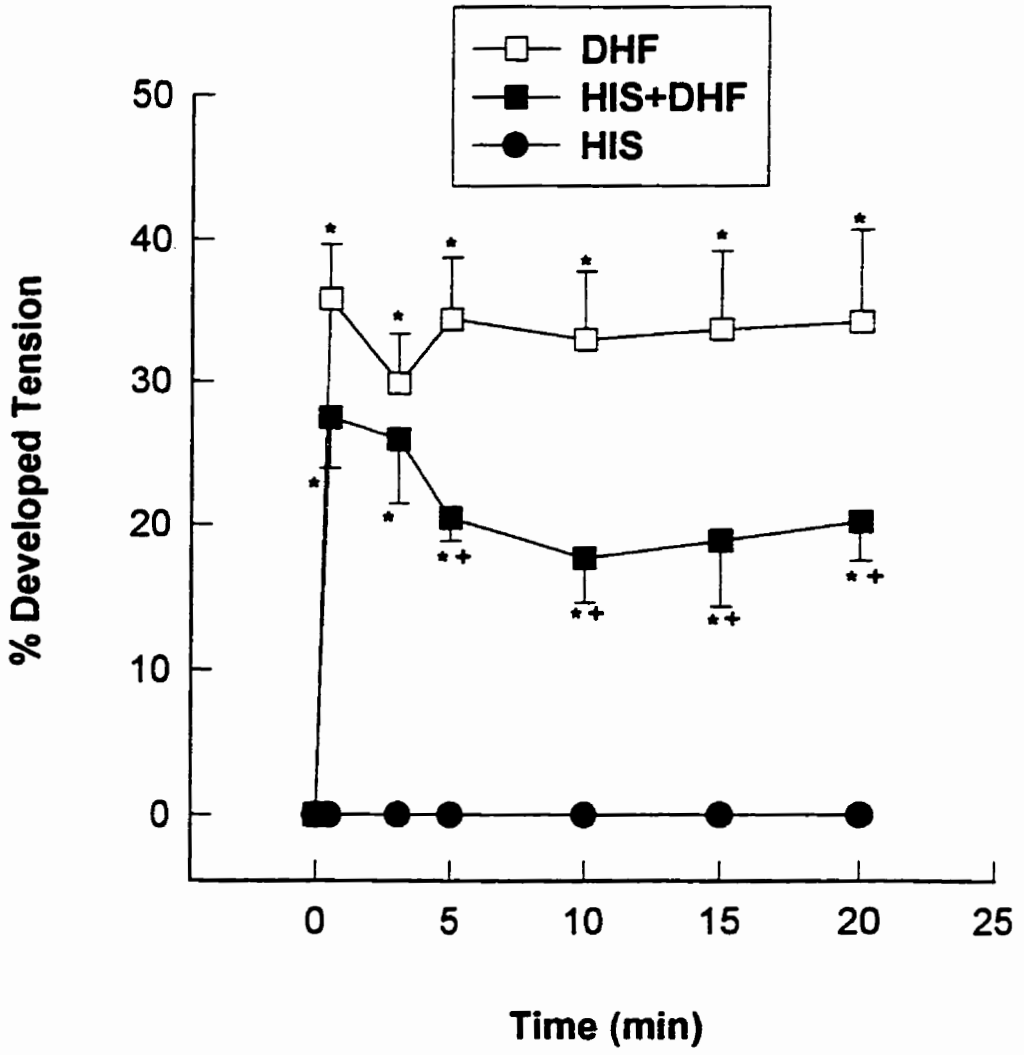

Figure 26: Effects of 2X concentration of DHF in absence or presence of histidine (HIS) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, DHF vs HIS (100 mM) + DHF.

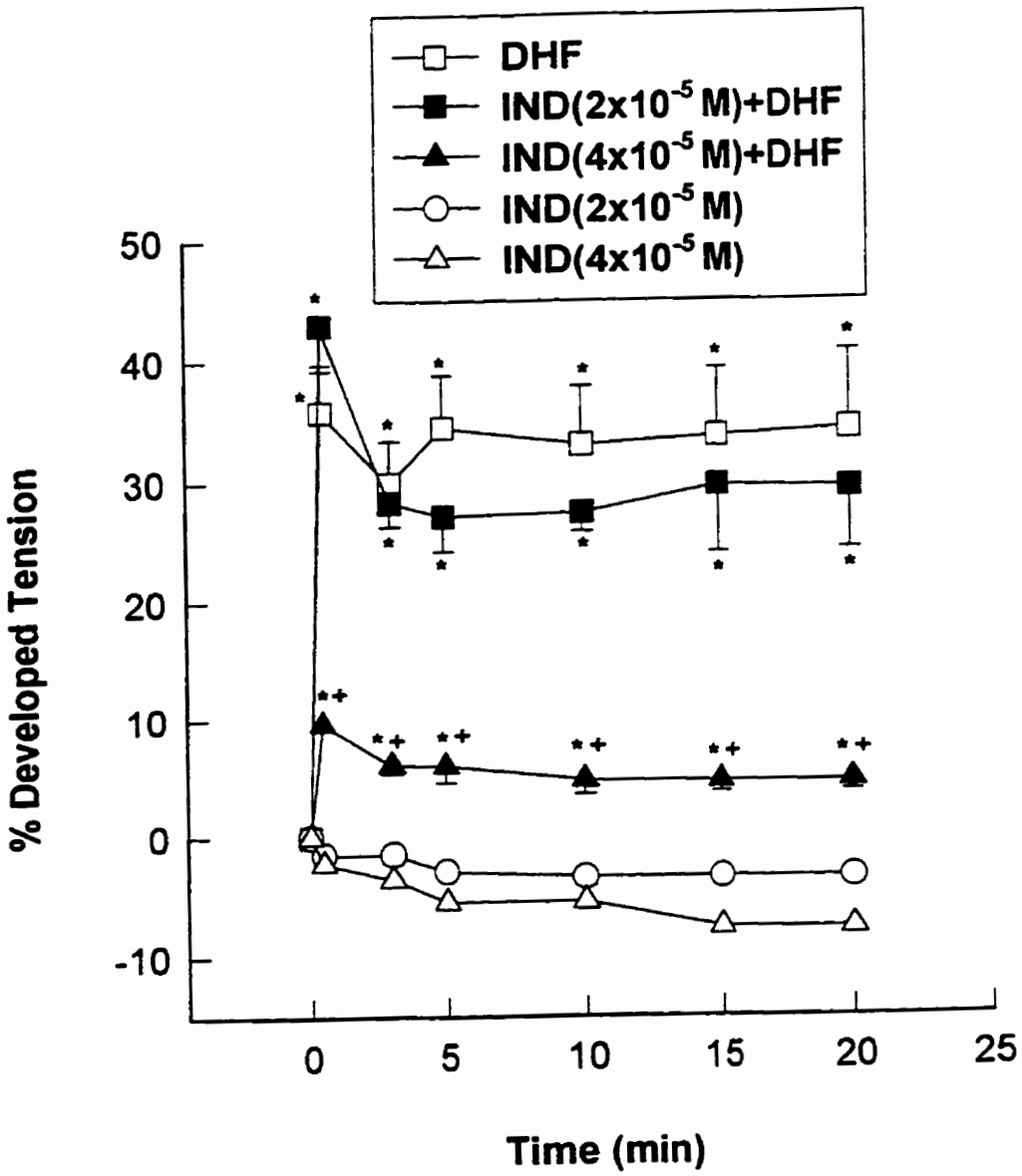

Figure 27: Effects of 2X concentration of DHF in absence or presence of two concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, DHF vs IND ($2x10^{-5}$ M) + DHF, or IND ($4x10^{-5}$ M) + DHF.

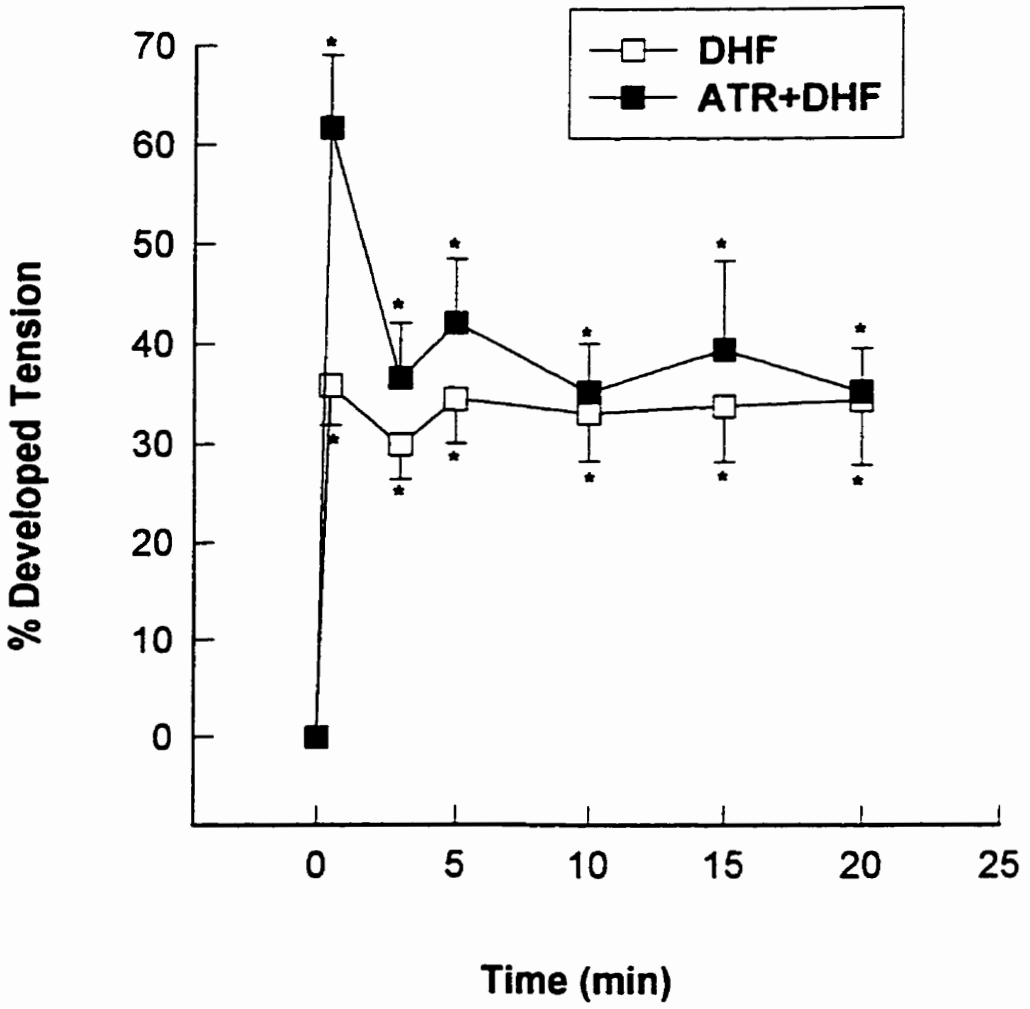

Figure 28: Effects of 2X concentration of DHF in absence or presence of atropine (ATR, 10^{-6} M)) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**

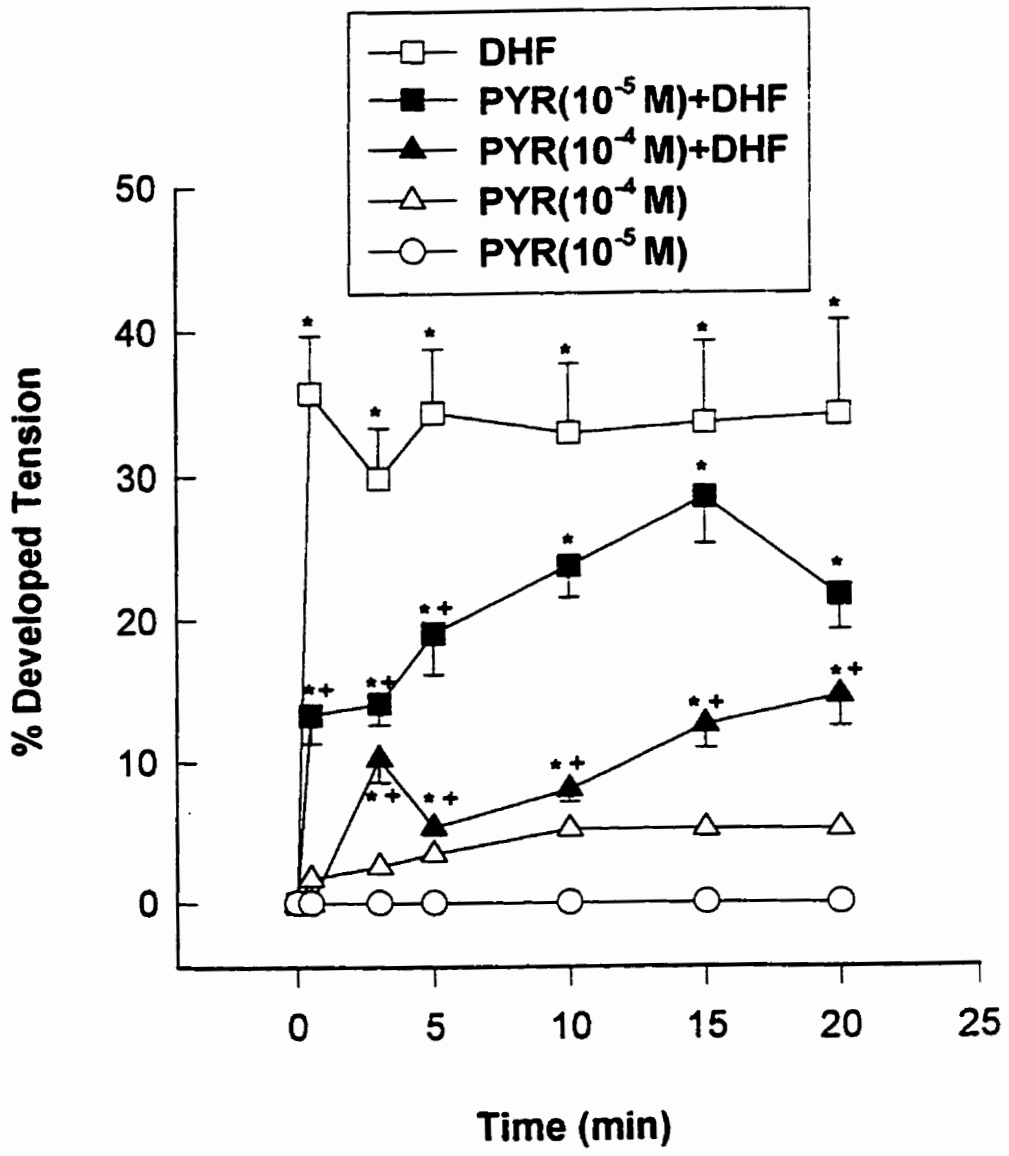

Figure 29: Effects of 2X concentration of DHF in absence and presence of two concentrations of pyrilamine (PYR) on ileum preparations.

Results are expressed as mean ± S.E.
*** P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+ P<0.05, DHF vs PYR (10^{-5} M) + DHF, or PYR (10^{-4} M) + DHF.

6.0 DISCUSSION

The focus of oxygen radicals and oxidants in biology has been on reactive oxygen metabolites (ROMs) as injurious agents. In the gastrointestinal tract, a role for ROMs has been postulated in various forms of ischemia/reperfusion and inflammatory bowel disease (Granger et al., 1986; Babbs, 1992). Ischemic/reperfusion injury has been the best studied model, wherein ROMs generated by an endogenous xanthine/xanthine oxidase (X/XO) reaction and from the respiratory bursts of neutrophils seem to be responsible for reperfusion injury, especially through the production of hydroxyl radical ($\cdot OH$). The present experimental data indicate that exogenously generated OFRs from X/XO, DHF/$FeCl_3$-ADP and also H_2O_2 can modulate the tone of isolated rat ileum. The results of these studies will be discussed further under specific subtitles.

6.1 Xanthine plus Xanthine Oxidase

6.1.1 Effects of Xanthine plus Xanthine Oxidase on Ileum

Xanthine and xanthine oxidase have been used by other investigators to generate ROMs (Prasad et al., 1989; Bharadwaj, 1997).

The results show that 1X, 2X and 4X concentrations of

X/XO produced relaxation of ileum. A concentration of 2X xanthine plus xanthine oxidase resulted in a maximal relaxant effect. A concentration of 4X elicited a response that was smaller than 2X concentration. One would have expected greater relaxation with 4X concentration of X/XO. This unexpected finding could be due to several reasons. Firstly, the higher concentrations of X/XO may have inhibited its cyclooxygenase enzyme with the loss of production of prostaglandins, and hence reduced relaxation. Secondly, it is possible that there is destruction of xanthine oxidase activity by OFRs.

A smaller response with 4X concentration of X/XO could also be due to high levels of OFR production, which may be cytotoxic and damaged the luminal epithelium or the underlying ileum smooth muscle. The cytotoxic effects of ROMs are well documented (Del Maestro et al., 1981; Thomas et al., 1985; Thomas et al., 1991). Xanthine/xanthine oxidase have been shown to have direct cytotoxic effects on cultured gastric mucosal cells (Hiroishi et al., 1987). Superoxide anions are known to cause cell damage (Heinle, 1984). Xanthine oxidase itself has been shown to alter myofibrillar sulfhydryl content and ATPase activity *in vitro* (Ventura et al., 1985). Therefore, high concentrations of X/XO may be directly altering the contractile apparatus of the ileum smooth muscle, resulting in a smaller response.

It may also be that the non-enzymatic sources of

antioxidants (Vit-E, Vit-C and β-carotene) are severely depressed or depleted in the presence of high concentrations of OFRs generated by the 4X concentration of X/XO. Therefore, increased cytotoxic effects on smooth muscle could be due to a decreased antioxidant reserve. Oxidants have been shown to directly inactivate enzymatic antioxidant defense systems. For example, H_2O_2 or a combination of $O_2^{\cdot -}$ and H_2O_2 can inactivate CuZn-SOD (Sinet and Garber, 1981). In addition, $O_2^{\cdot -}$ can increase the susceptibility of cells to H_2O_2 by converting catalase to an inactive form (Shimizu et al., 1984).

In the literature, there is no study on the effects of X/XO on ileum; however, some investigators have studied the effect of X/XO on smooth muscles from other sources (Del Maestro et al., 1981; Katusic and Vanhoutte, 1989). Smooth muscle relaxation in response to X/XO has been reported in rabbit arteries, dog coronary arteries and rat pulmonary arteries (Silin et al., 1985; Wolin and Belloni, 1985; Vanhoutte and Rubanyi, 1985; Archer et al., 1989). Xanthine plus xanthine oxidase has also been reported to contract rat pulmonary arteries and aorta (Del Maestro et al., 1981; Katusic and Vanhoutte, 1989). This discrepancy may be attributed to the tissue differences.

To investigate the toxic effect of X/XO, the influence of the X/XO on ACh-induced contraction of rat ileum was studied. Acetylcholine-induced contraction was greater in 1X

and 2X concentrations of X/XO-treated groups than in control groups. There is considerable evidence that suggests the enhancement of contractile activity of smooth muscle that is chronically exposed to H_2O_2 *in vivo* (Prasad and Gupta, 1993). They reported that hyperresponsiveness of the airways to ACh may be related to epithelial dysfunction. Therefore, the increased sensitivity of ileum smooth muscle to ACh could be due to damage of the epithelium.

Although 1X and 2X concentrations of X/XO increased the sensitivity of ileum to ACh, 4X concentration of X/XO reduced muscle responsiveness to ACh. It is possible that the biologic activity of ACh could be destroyed by oxidation via X/XO. Also high amounts of OFRs generated by 4X concentration of X/XO might damage the cell membrane and alter the coupling of the muscarinic receptor to its effector system. Similar findings were reported by Moummi et al., (1991b), who found that pretreatment of guinea pig ileum with oxidants reduced the subsequent contractile response to carbachol. Hiero et al., (1989) also showed that the ROMs may contribute to the inhibition of agonist-stimulated contraction of human colon smooth muscle *in vitro*.

Another explanation for reduced contractile responses to ACh with 4X concentration of X/XO may involve intracellular damage by oxidants as proposed by Goldhaber et al., (1989). They investigated the effects of exogenous free radicals on electromechanical function and metabolism

in isolated rabbit and guinea pig ventricle, and found that free radicals may contribute to electrophysiologic abnormalities and contractile dysfunction by inhibiting glycolysis and oxidative phosphorylation.

6.1.2 Oxygen Metabolites Generated by the Xanthine plus Xanthine Oxidase System and Ileal Relaxation

The results showed that xanthine plus xanthine oxidase-induced relaxation of ileum does not appear to be mediated through superoxide anion because superoxide dismutase (SOD), which metabolizes $O_2^{\cdot -}$ to H_2O_2 could not abolish the relaxation.

In the literature, there are no reports on the effects of X/XO on ileal preparations; however, Rhoades et al., (1988; 1990) have shown that superoxide dismutase had minimal effects on xanthine plus xanthine oxidase-induced responses of rat pulmonary arteries. In contrast to the above, using rabbit aorta, Bharadwaj and Prasad, (1994) showed that X/XO induced contraction of aorta, and SOD reduced the effects of X/XO.

The protective effect of SOD against oxidant injury has been questioned by some investigators because they either did not find SOD to be protective, or they found SOD actually increased the level of X/XO-induced cellular injury. The discrepancies that exist in the literature concerning the protective effect of SOD on X/XO-induced oxidant injury may

in part have resulted from using impure preparations of SOD. A report by Yagoda et al., (1991) indicates that some commercial preparations of SOD were contaminated with endotoxin. As well, the possibility exists that the lack of effect of superoxide dismutase may reflect a failure of its endocytosis, which is required before it can protect against H_2O_2-induced injury (Reilly et al., 1991).

The inability of SOD to prevent the relaxant effect of X/XO could also be due to the fact that superoxide dismutase metabolizes $O_2^{\cdot -}$ to H_2O_2 (Southorn and Powis, 1988). The amount of H_2O_2 produced under these circumstances may have been sufficient to induce relaxation of the ileum. In this investigation, however, xanthine plus xanthine oxidase-induced relaxation does not appear to be mediated through hydrogen peroxide because catalase did not reduce the relaxation of ileum. One possibility is that generation of H_2O_2 exceeded the catalytic capacity of catalase. Another possibility is that H_2O_2 crosses the cell membrane and produces hydroxyl radicals.

The results showed that neither $O_2^{\cdot -}$ nor H_2O_2 is responsible for relaxation of ileum. It should be noted that although the xanthine-xanthine oxidase system can generate both $O_2^{\cdot -}$ and H_2O_2, if there is insufficient enzymatic protection, these two species can interact via the process known as the Haber-Weiss reaction to generate highly reactive intermediates, the most important of these being the hydroxyl

radical ($\cdot OH$) (Kellogg and Fridovich, 1975).

Therefore, it is possible that hydroxyl radicals are generated. The results suggest that X/XO-induced relaxation of ileum is mediated through hydroxyl radical, because two powerful hydroxyl radical scavengers, dimethylthiourea and mannitol, reduced the relaxation of ileum.

In the literature, there are no studies on the role of hydroxyl radical in X/XO-induced relaxation of ileum; however, studies with other smooth muscle such as rabbit pulmonary arteries demonstrated that responses induced by the addition of xanthine plus xanthine oxidase are attributed to the hydroxyl radical (Del Maestro et al., 1981). Hydroxyl radicals produced as a by-product of $O_2^{\cdot -}$ and H_2O_2 via the Haber-Weiss reaction have been shown to be the most injurious oxygen species involved in cellular injury of small intestinal epithelial cell induced by xanthine oxidase (Thomas et al., 1991). Studies on pial arterioles of the mouse in which OFRs were generated by the xanthine oxidase reaction also suggested that the radical producing most of the relaxation in these blood vessels was the hydroxyl radical (Rosenblum, 1983).

However, the formation of hydroxyl radical from superoxide and hydrogen peroxide requires the presence of transition metal ions, the most significant of which are iron ions (Thomas et al., 1985). The results of this study indicate that pretreatment with the iron chelator

deferoxamine provided significant protection against X/XO-induced relaxation. This finding indicates that iron is involved in X/XO-mediated relaxation of ileum, likely as a result of its participation in the Haber-Weiss reaction.

Iron is present within cells as low molecular weight iron chelates, heme-associated iron and ferritin-bound iron (Bacon and Tavill, 1984). A role of the low molecular weight chelatable iron pool and heme associated iron was implicated in the intracellular formation of hydroxyl radical (Kvietys et al., 1989). The most effective mobilizers of ferritin-bound iron include xanthine oxidase and the superoxide radical (Green and Mazur 1957; Thomas et al., 1985; Fridovich, 1988).

A role for intracellular iron and intracellular formation of the hydroxyl radicals has been implicated in H_2O_2-mediated bacterial killing (Repine et al., 1981), neutrophil-mediated endothelial cell injury (Gannon et al., 1987) and xanthine oxidase-induced injury to endothelial cells (Kvietys et al., 1989). Repine et al., (1981) showed that increasing the intracellular iron concentration in Staphylococcus aureus enhanced their subsequent susceptibility to killing by H_2O_2. Similarly, Gannon et al., (1987) demonstrated that neutrophil-mediated endothelial cell injury can be prevented by pretreatment of the endothelial cells with deferoxamine, an iron chelator. Furthermore, Kvietys et al., (1989) showed that pretreatment of the

endothelial cells with deferoxamine protected the endothelial cells from the cytotoxic effects of the hypoxanthine/xanthine oxidase reaction mixture or H_2O_2.

Deferoxamine at appropriate concentration has been shown to be protective against xanthine/xanthine oxidase-induced intestinal injury (Thomas et al., 1991). Deferoxamine reduced the creatine kinase release from post-ischemic isolated rabbit hearts and enhanced functional and metabolic recovery as well (McCord and Omar, 1993). Protection by deferoxamine has also been shown in several *in vivo* and *in vitro* studies. *In vivo*, a protective effect has been shown with deferoxamine in heart (Asbeck, 1990). Deferoxamine prevented ventricular fibrillation and normalized contractility in reperfused iron-loaded hearts (Asbeck, 1990). Deferoxamine also afforded protection against ischemia/reperfusion-induced gastric ulceration (Smith et al., 1987).

However, there are some concerns about the use of deferoxamine, because besides its iron-chelating effect, it has the capacity to react with superoxide to form a relatively stable nitroxide (Asbeck, 1990). However, the reaction of deferoxamine with $O_2^{\cdot -}$ is very slow (Asbeck, 1990), and is unlikely to influence the interpretation of experiments in which the chelator is used. Deferoxamine also reacts with hydroxyl radical and it may be suggested that the interaction could be a source of error in the explanation of results using the chelator. However, to form $^{\cdot}OH$, which then

might react with deferoxamine, iron in the ferrous oxidation state is needed. Although deferoxamine binds the Fe^{2+} ion weakly, if at all, the chelator strongly promotes the oxidation of the ferrous ion, thus maintaining iron in the ferric state and inhibiting its reaction with H_2O_2.

The results of this study indicate that in the presence of histidine, a singlet oxygen ($^1O_2$) scavenger, X/XO-induced relaxation was significantly reduced. The protective effect of histidine was concentration-dependent and was comparable to classical free radical scavengers such as mannitol. These results suggest that singlet oxygen may be one of the free radicals (in addition to ·OH radical) involved in X/XO-induced relaxation.

Singlet oxygen is not a radical, but rather an excited state of oxygen which results from the promotion of an electron to its higher energy orbital. Its high reactivity can damage lipids and other constituents of biological membranes and can inactivate enzymes, cause DNA damage and oxidation of mitochondrial components (Kukreja and Hess, 1992; Wefers, 1987). The observed singlet oxygen-induced inhibition of Na^+K^+-ATPase, possibly along with inactivation of Ca^{2+}-ATPase in SR, may be a link between free radical action and disturbed cellular Ca^{2+} homeostasis. The subsequent membrane changes as well as ion homeostasis may result in failure of excitation-contraction coupling as has been suggested in myocardial cell by Vinnikowa et al., (1992) and

Kukreja et al., (1991).

Singlet oxygen can be generated from superoxide anion in the xanthine/xanthine oxidase system (Duran, 1982). In fact, one of the toxic effects of $O_2^{\cdot -}$ generating systems is their ability to produce singlet oxygen either in the non-enzymatic dismutation reaction of $O_2^{\cdot -}$ or in the metal catalyzed Haber-Weiss reaction (Kukreja and Hess, 1992; Duran, 1982; Fridovich, 1975).

In the literature, there is no study on the effects of singlet oxygen on ileum; however, some investigators studied the effects of $^1O_2$ in other tissues (Kukreja and Hess, 1992; Kukreja et al., 1992). Singlet oxygen has been shown to produce a negative inotropic effect on isolated papillary muscle (Kukreja and Hess, 1992; Kukreja et al., 1992). Singlet oxygen derived from activated neutrophils has also been implicated in causing myocardial and peripheral vascular depression (Kukreja and Hess, 1992).

In summary, the partial protection offered by all the antioxidants used in this study indicate that other possibilities are involved in the relaxation process. It has been proposed that cell injury may not be mediated by the hydroxyl radical, but by an intermediate, higher oxidation state of iron (Fe^{4+}) or ferryl species formed by the Fenton reaction (Sutton and Winterbourn, 1989). This ferryl species is a potent hydroxyl-like oxidant and could readily lead to cytotoxicity. Another possibility is the formation of

hydroxyl radical at places inaccessible to scavengers, where iron can promote the generation of hydroxyl radical.

6.1.3 Mechanisms of Xanthine plus Xanthine Oxidase-Induced Relaxation of Ileum

The results show that the cyclooxygenase inhibitor indomethacin reduced X/XO-induced relaxation of ileum, suggesting that cyclooxygenase-derived relaxing metabolites are involved in the X/XO-induced relaxation. Therefore, oxygen free radicals may be stimulating prostaglandin (PG) release from smooth muscle cells, thus producing relaxation.

The links between oxygen free radicals and PGs are supported by several studies (Lewis et al., 1988; Otamiri et al., 1988; Bern et al., 1989; Karayalcin et al., 1990). Reactive oxygen metabolites stimulated PGE_2 and/or PGI_2 production in mast cells, fibroblasts and endothelial cells (Lewis et al., 1988). The mechanism of stimulation is unclear, but in the endothelial cells it seems to involve gating Ca^{2+} across the cell membrane either as the result of a receptor-mediated event or secondary to lipid peroxidation of the cell membrane, making it more permeable to Ca^{2+} with subsequent liberation of arachidonic acid from membrane stores (Lewis et al., 1988).

Reactive oxygen metabolites are also able to stimulate phospholipase A_2 and 5-lipoxygenase directly, and thus may promote formation of prostaglandins (Otamiri et al., 1988).

Oxidants generated by xanthine and xanthine oxidase have been shown to increase PGE_2 and prostacyclin tissue levels (Bern et al., 1989; Karayalcin et al., 1990). Also, indomethacin has been shown to reduce the effects of X/XO (Gupta and Prasad, 1992; Bharadwaj and Prasad, 1994) and to prevent hydroxyl radical-induced relaxation in an airway smooth muscle preparation (Prasad and Gupta, 1993).

The concern relating to the use of indomethacin is that the antioxidant properties of indomethacin may involve interaction *in vitro* with $^1O_2$ and $\cdot OH$ (Prasad and Laxdal, 1994). However, the concentration used in this study has very weak antioxidant activity, and it is generally thought to act mainly by inhibiting cyclooxygenase activity (Prasad and Laxdal, 1994).

The relaxation of ileum by X/XO in rat might be due to the release of nitric oxide (NO) by OFRs. Data obtained from these studies in which rat ileum is pretreated with L-NMMA, an inhibitor of NO synthases, suggest that NO is partially responsible for relaxation. However, the inhibitory action was transient.

Both the constitutive and the inducible isoforms of nitric oxide synthases were reported to be present in the rat small intestine (Dignass et al., 1995). Nitric oxide can be synthesized in the intestine by endothelial cells, neurons, macrophages, epithelial cells and smooth muscle cells (Nichols et al., 1993a; Nichols et al., 1993b; Dignass et

al., 1995; Kanada et al., 1992; Nathan, 1992).

Nitric oxide was suggested to play a role in NANC inhibitory responses in the rat ileum (Kanada et al., 1992). The ROMs, by gaining access directly to the nerve endings, may induce injury to the myenteric plexus, releasing neurotransmitter (s) as part of the destruction of nerve terminal (Moummi et al., 1991a; Moummi et al., 1991b). Also, antioxidants have been shown to protect neuronal cells *in vitro* from toxicity (Miyamoto et al., 1989).

The link between oxygen free radicals and NO production could be supported by the fact that oxygen radicals are required for the synthesis of nitric oxide by NO synthase as demonstrated by inhibition of NO formation by oxygen radical scavengers (Mittal, 1995). Oxygen radicals and hydrogen peroxide participate in the catalytic conversion of L-arginine to nitric oxide by nitric oxide synthase in the presence of calcium ion (Mittal, 1995). The exact mechanism(s) whereby oxygen radicals participate in NO formation are not known, but Mittal, (1995) proposed that the hydrogen peroxide could peroxidatively oxidize the guanidino nitrogen of L-arginine to NO by NO synthase.

Nitric oxide-induced relaxation is mediated through cGMP (Moncada et al., 1991; Kanada et al., 1992). Nitric oxide stimulates the enzyme guanylate cyclase, which converts GTP to cGMP (Moncada et al., 1991). Cyclic guanosine monophosphate activates a cGMP-dependent protein kinase which

catalyzes the phosphorylation of phospholamban, a regulator of sarcoplasmic Ca^{2+}-ATPase (Moncada et al., 1991), thus decreasing intracellular calcium. A decrease of intracellular calcium results in a loss of smooth muscle tone.

Guanylate cyclase may also be regulated by free radicals. Hydroxyl radical has been shown to activate guanylate cyclase (Mittal and Murad, 1977; Waldman and Murad, 1987). Mittal, (1995) has reported that oxygen radicals stimulate cytosolic guanylate cyclase. Also, arachidonic metabolism and prostaglandin formation can increase levels of hydroxyl radical, and these processes are associated with elevated levels of cGMP as well as increases in guanylate cyclase activity (Waldman and Murad, 1987).

However, in the present study, methylene blue, an inhibitor of guanylate cyclase (Martin et al., 1985) did not reduce the X/XO-induced relaxation, indicating that relaxation is not mediated through the cGMP pathway. The discrepancy between the effects of L-NMMA and methylene blue could have been due to the fact that methylene blue can generate $O_2^{\cdot -}$ which in turn can yield $^{\cdot}OH$ (McCord and Fridovich, 1970; Rand and Li, 1995). The $^{\cdot}OH$ produced may result in further relaxation of ileum.

The results presented show that ATP-sensitive potassium channels are involved in the relaxation-induced by X/XO. Although the transmembrane potential in the ileum smooth muscle was not measured, the observation that a high

concentration of glibenclamide decreased the X/XO-induced relaxation suggests that activation of the ATP-sensitive K^+ channel plays a role in X/XO-induced relaxation. Adenosine triphosphate-sensitive K^+ channels are known to be present in rat ileal smooth muscle cells and are blocked by glibenclamide (Frank et al., 1994).

The link between OFRs and ATP-sensitive potassium channels can be supported by some studies. Goldhaber et al., (1989) reported that the OFRs generated by xanthine and xanthine oxidase or H_2O_2 can activate K^+-ATP channels. Direct evidence for opening of K^+-ATP channels was also obtained from single channel current recordings in which OFRs increased the channel activity, and glibenclamide effectively prevented the opening of the channels (Tokube et al., 1996).

In the literature, there is no study on the role of ATP-sensitive potassium channels in X/XO-induced relaxation of ileum; however, OFRs-induced relaxation of other smooth muscle such as aorta and trachea has been shown to be mediated partly through ATP-sensitive K^+ channels (Bharadwaj and Prasad, 1997; Gupta and Prasad, 1992).

In addition to the mechanisms already discussed, the direct effect of OFRs generated by X/XO on smooth muscle cells should not be ruled out. Xanthine/xanthine oxidase has been shown to have direct cytotoxic effects on cultured gastric mucosal cells (Hiroishi et al., 1987). Also, oxidants can induce cellular injury (Grisham and Granger, 1988) and

affect smooth muscle contractility (Moummi et al., 1991a; Wright and Low, 1989). Furthermore, ·OH produced as a by-product of $O_2^{\cdot -}$ and H_2O_2 via xanthine oxidase has been shown to be the most injurious oxygen species involved in cellular injury of IEC-18 small intestinal epithelial monolayers (Thomas et al., 1991).

6.1.4 Effect of Xanthine plus Xanthine Oxidase on Spontaneous Activity of Ileum

The results show that X/XO decreased the amplitude of spontaneous activity of ileum, and indomethacin abolished the inhibitory effects of X/XO on spontaneous activity of rat ileum. It is therefore suggested that prostaglandins are involved in the reduction of spontaneous activity. In support of these data, Kubota et al., (1982) have shown that all prostaglandins (PGs) suppressed both the spontaneous discharges and the mechanical activity of the smooth muscle cells, and raised the threshold for the generation of action potentials. The precise mechanisms involved in the inhibitory actions of PGs on excitability of the smooth muscle membrane are unknown; however, it has been reported that PGs couple with extracellular Ca^{2+} at activated nerve terminals, thereby reducing the amount of transmitter released by adrenergic or cholinergic nerve fibers (Ito and Tajima, 1979; Ito and Tajima, 1981). Such interactions between PGs and extracellular Ca^{2+} could explain the effects of PGs on the

threshold for evoked action potentials and on spontaneously generated action potentials of smooth muscle cells (Kubota et al., 1982). Indomethacin evokes spontaneous membrane activity of smooth muscle cells by an inhibitory action on PG biosynthesis rather than by a direct action on the smooth muscle cells (Kubota et al., 1982). It is also already known that changes in PG synthesis correlate with changes in motility of the ileum (Sanders, 1978).

In the literature, there are some studies which show that spontaneous activity can be affected by OFRs (Bielefeldt and Conklin, 1997; Van der Vliet et al., 1989). Hypoxia-reoxygenation through the generation of reactive oxygen species and disruption in calcium homeostasis significantly altered intestinal motility (Bielefeldt and Conklin, 1997). During hypoxia, the frequency of the spontaneous contractions and the resting tension decreased (Bielefeldt and Conklin, 1997). Also, an *in vitro* study by Van der Vliet et al., (1989) showed that H_2O_2 and cumene hydroperoxide diminished the spontaneous motility of rat intestinal longitudinal smooth muscle.

6.2 Hydrogen Peroxide

6.2.1 Effects of Hydrogen Peroxide on Ileum Preparations

The results show that hydrogen peroxide produced a biphasic response in rat ileum and the effects were concentration-dependent. The biphasic response was composed

of a transient contraction followed by relaxation. Differences in the type of response to H_2O_2 may be due to a contrast in receptor number and / or affinity for H_2O_2, dissimilarity in second messenger systems, or to a variation in mediators released.

In support of this result, Van der Vliet et al., (1989), documented a biphasic response in rat small intestine upon direct exposure to H_2O_2 , and the phases that occurred were similar to those reported here (i.e., contraction was followed by relaxation).

In contrast to these results, hydrogen peroxide has been reported to contract other smooth muscles such as airway smooth muscle of guinea pig and rat (Khan et al., 1990; Szarek and Schmidt, 1990); to relax the rabbit tracheal smooth muscle (Gupta and Prasad, 1992) and to produce biphasic responses in the opposite order of this study, consisting of transient relaxation followed by contraction of rabbit aortic preparations (Bharadwaj and Prasad, 1995). These dissimilarities in results may be due to tissue differences.

The biphasic responses of rat ileum are mediated through H_2O_2, because catalase prevented both contraction and relaxation. Also, in the present study, two hydroxyl radical scavengers, dimethylthiourea and mannitol, prevented the effects of H_2O_2-induced biphasic response, suggesting that hydroxyl radical ($^{\cdot}OH$) plays an important role in muscle

contraction and relaxation. A possible explanation for this is that hydrogen peroxide is known to cross membranes easily and interact with reduced state transition metal ions to form hydroxyl radical.

There are, however, some contrary findings related to the role of hydroxyl radical in the H_2O_2-induced biphasic response. Van der Vliet et al., (1989) reported that the hydroxyl radical scavenger mannitol had no inhibitory effect on H_2O_2-induced responses. This apparent discrepancy may be due to the concentration of mannitol used. They used a concentration of mannitol (10 mM) which was smaller than that used in this study. Because of the site-specific generation of hydroxyl radical (Freeman and Crapo, 1982) and the extremely high reactivity of $\cdot OH$, it is difficult to scavenge hydroxyl radical effectively in the living tissues, unless the specific scavenger reaches very high concentrations.

6.2.2 Mechanisms of Hydrogen Peroxide-Induced Biphasic Responses

Further investigation into the mechanisms of H_2O_2-induced contraction and relaxation revealed that the H_2O_2-induced biphasic response may be a prostaglandin (PG)-mediated phenomenon, because indomethacin prevented the H_2O_2-induced contraction and relaxation. These results suggest that H_2O_2 may trigger the release of PGs to cause contraction and relaxation.

Van der Vliet et al., (1989) have also hypothesized that the effects of H_2O_2 on intestine could be due to release of PGs, but they did not do further work in this area. Others have noted that hydrogen peroxide stimulates prostaglandin production in the rat colon (Karayalcin et al., 1990). Finally, Moummi and coworkers (1991b), reported that the reactive oxygen metabolite (H_2O_2) caused contraction of resting guinea pig ileal smooth muscle strips via release of prostaglandins.

Hydrogen peroxide has also been shown to stimulate the release of arachidonic acid in other smooth muscles such as trachea and aorta (Gupta and Prasad, 1992; Bharadwaj and Prasad, 1994). Hydrogen peroxide produced contraction in guinea pig and rat tracheal preparations and indomethacin inhibited H_2O_2-induced contraction, suggesting that H_2O_2-induced contraction may be mediated by PGs (Rhoden and Barnes, 1989; Szarek and Schmidt, 1990).

There are several potential mechanisms whereby H_2O_2 might stimulate production of PGs in the intestine. Peroxides are important cofactors for prostaglandin biosynthesis, as demonstrated by the effect of exogenous addition of either H_2O_2 or lipid peroxides to tissues (Weiss et al., 1979; Hemler and Lands, 1980). Hydrogen peroxide has been shown to be a potent stimulus for PGE_2 and prostacyclin production (Karayalcin et al., 1990). Also, oxidants increase phospholipase A_2 activity, which in turn increases the

release of arachidonic acid and synthesis of PGs (Chakraborti et al., 1989).

Furthermore, there is another mechanism for peroxide stimulation of PG production: the activation of inflammatory or mesenchymal cells. Hydrogen peroxide stimulates PGE_2 and / or PGI_2 production in mast cells (Stendahel et al., 1983), fibroblasts (Taylor et al., 1983) and cultured endothelial cells (Harlan and Callahan, 1984; Lewis et al., 1988). The mechanism of this stimulation is unclear, but in the endothelial cells it seems to involve gating of Ca^{2+} across the cell membrane either as a result of a receptor-mediated event or secondary to lipid peroxidation of the cell membrane, making it permeable to Ca^{2+}, with subsequent liberation of arachidonic acid from membrane stores (Lewis et al., 1988). Increased PGs in the lamina propria might initiate changes in smooth muscle function.

Hydrogen peroxide-induced relaxation has been shown to be associated with a release of nitric oxide and stimulation of guanylate cyclase by this peroxide (Gupta and Prasad, 1992; Bharadwaj and Prasad, 1995). This may also account for the relaxation found in this study. However, the experimental results presented here demonstrate that pretreatment of ileum with L-NMMA (an NO synthase inhibitor) or methylene blue did not reduce the relaxant effect of H_2O_2 on the ileum, suggesting no involvement of NO or cGMP.

Glibenclamide, an ATP-sensitive K^+ channel blocker, was

found to be ineffective in decreasing the relaxation of ileum by H_2O_2; however, it reduced the contraction produced by H_2O_2 significantly. In support to this finding, Delaey and Van de Voorde, (1995) have shown that sulfonylureas like glibenclamide blocked contractions induced by prostaglandin $F_2\alpha$, PGE_2 and the thromboxane A_2 mimetics on rat aorta and exerted a specific inhibitory influence on prostanoid-induced contractions (Delaey and Van de Voorde, 1995).

The lack of inhibitory effect of atropine in this study suggests that ACh is not involved in the oxidant-induced response. Finally, the lack of efficacy of the antihistamine pyrilamine argues against a contributory role for the proinflammatory mediator histamine on the contractile response to the oxidant.

The results also do not rule out the possibility that H_2O_2 might directly stimulate the smooth muscle cells. H_2O_2 stimulates the production of inositol triphosphate in smooth muscle cells (Korchak et al., 1985). The oxidative challenge by H_2O_2 causes a profound imbalance of calcium homeostasis in smooth muscle cell (Roveri et al., 1992). It is possible that H_2O_2 acts directly on ileum smooth muscle membranes to stimulate the production of inositol triphosphate (Roveri et al., 1992; Korchak et al., 1985). An increase in intracellular inositol triphosphate causes the release of calcium from intracellular stores (sarcoplasmic reticulum) thereby eliciting a contractile response (Berne and Levy,

1993). There is no study on the direct effect of H_2O_2 on ileum motility, however, hydrogen peroxide has been shown to produce a direct effect on other smooth muscle such as vascular smooth muscle (Tate et al. 1984; Sheehan et al., 1993; Wolin et al., 1985).

6.3 Dihydroxy Fumaric Acid plus Ferric Chloride and Adenosine Diphosphate

6.3.1 Effects of Oxygen Radicals Generated by Dihydroxy Fumaric Acid plus Ferric Chloride and Adenosine Diphosphate on Ileum Preparations

The combination of dihydroxy fumaric acid plus ferric chloride and adenosine diphosphate has been used by other investigators to generate hydroxyl radical (·OH) (Prasad and Gupta, 1993; Bharadwaj and Prasad, 1997). The concentrations used in this study to generate ·OH have been formulated on the concentrations used by these investigators (Prasad and Gupta, 1993; Bharadwaj and Prasad, 1997); however, in addition, one higher concentration of DHF/$FeCl_3$-ADP was also employed in this study.

The results showed that the combination of dihydroxy fumaric acid, ferric chloride and adenosine diphosphate produced concentration-dependent contraction of ileum. The addition of DHF/$FeCl_3$-ADP to the bathing solution of rat ileum produced a maximal contraction at 0.5-1 minute, after which the tension reduced slowly. The slow recovery in

tension could be a result of a decrease in levels of OFRs produced by DHF/$FeCl_3$-ADP. In fact, Todoki et al., (1992), using electron spin resonance spectroscopy, documented the time-course of ·OH generation by DHF/$FeCl_3$-ADP. They documented that the signal intensity of ·OH adduct formation reached maximum response immediately, showed a sharp decline (70%) during the first 30 minutes, and a further decline of 82% at 90 minutes. Therefore, these observations may explain the nature of DHF/$FeCl_3$-ADP-induced contraction of ileum in this experiment.

The series of chemical reactions between dihydroxy fumaric acid plus ferric chloride and adenosine diphosphate generate all three oxygen radical species (superoxide anion, hydrogen peroxide and the hydroxyl radical). However, the results presented show that the contraction produced by the action of DHF/$FeCl_3$-ADP on ileum are not mediated by superoxide anion or hydrogen peroxide, since neither superoxide dismutase (a $O_2^{\cdot -}$ metabolizer) nor catalase (a H_2O_2 scavenger) reduced the contraction.

Hydroxyl radicals are also generated from autooxidizing DHF in the presence of $FeCl_3$-ADP, as shown in electron spin resonance (Todoki et al., 1992). In this study, dimethylthiourea (Prasad et al., 1994) and mannitol, two powerful hydroxyl radical scavengers, reduced the contractile response of the tissue to DHF/$FeCl_3$-ADP, suggesting that hydroxyl radical is involved in the contraction induced by

DHF/$FeCl_3$-ADP. The contractile effect is only partially mediated through the ·OH; however, because the contraction is only partially abolished. Inability of these two scavengers to completely inhibit contraction may be due to generation of very high concentration of hydroxyl radical which may overwhelm the metabolizing ability of the DMTU and mannitol at the concentrations used in this study, or may be due to the generation of singlet oxygen.

The direct proof of singlet oxygen ($^1O_2$) generation is currently lacking; however, one of the nonenzymatic sources of singlet oxygen production has been reported to be spontaneous dismutation of superoxide anion (Kukreja and Hess, 1992). Also, singlet oxygen can be generated by the enzymatic reactions of lipooxygenase and prostaglandin hydroperoxidase activity of PGH synthase (Duran, 1982; Krinsky, 1979) and hydroperoxides have been shown to activate PGH synthases (Marshall and Lands, 1986). It is therefore possible that addition of DHF/$FeCl_3$-ADP produces $^1O_2$, and the remaining contraction could be attributable to the actions of singlet oxygen. The results of this study indicate that in the presence of histidine, a $^1O_2$ scavenger, the DHF/$FeCl_3$-ADP-induced contraction was significantly reduced, supporting the role of $^1O_2$ in DHF/$FeCl_3$-ADP-induced contraction of ileum. This does not contradict the presence and participation of other oxygen intermediates, because $^1O_2$ is a side product of most pathways of free radical generation and interaction.

In the literature, there is no study on the effect of singlet oxygen on ileum; however, some investigators have studied the effect of $^1O_2$ on other tissues (Fadel et al., 1995; Kukreja et al., 1991; Vinnikowa et al., 1992). Singlet oxygen has been shown to cause cerebral vasospasm (Fadel et al., 1995). Kukreja et al., (1991) demonstrated that singlet oxygen inhibits Ca^{2+} uptake and depresses Ca^{2+}-ATPase activity in isolated canine cardiac sarcoplasmic reticulum. Singlet oxygen was also able to inhibit an important sarcolemmal enzyme, Na^+-K^+-ATPase (Vinnikowa et al., 1992). Inhibition of Na^+-K^+-ATPase can be responsible for the onset of Ca^{2+}-overload with $^1O_2$ in ischemic-reperfused hearts. Thus, $^1O_2$-induced inhibition of Na^+-K^+-ATPase, possibly along with inactivation of Ca^{2+}-ATPase in SR, may be a link between free radical action and disturbed cellular Ca^{2+}-homeostasis.

6.3.2 Mechanisms of Dihydroxy Fumaric Acid plus Ferric Chloride and Adenosine Diphosphate-Induced Contraction of Ileum

The contractile response elicited by DHF/$FeCl_3$-ADP could be attributed to various factors. It may be mediated through the synthesis and release of arachidonic acid metabolites. The presence of oxygen free radicals may result in altered prostanoid ratios, favoring greater generation of constrictor prostanoids over relaxant prostanoids.

The results presented show that the contractile effects

of DHF/$FeCl_3$-ADP are inhibited by indomethacin, an inhibitor of PG synthesis, suggesting that contraction may be partly mediated through the release of prostaglandins from the luminal epithelium. Thus, these results support the involvement of contractile prostaglandins in DHF/$FeCl_3$-ADP-induced contraction of ileum smooth muscle.

Prostaglandins are generated whenever membrane damage occurs (Salmon and Flower, 1979), whether it be neural injury or injury to cells in lamina propria or epithelial cells (Berschneider and Powell, 1992). The hydroxyl radical has been demonstrated to both stimulate and inhibit the synthesis of prostaglandins (Deby and Deby-Dupont, 1981). Oxidants have also been shown to increase phospholipase A_2 activity, which in turn increases the synthesis of prostaglandins (Chakraborti et al., 1989). In previous studies involving rat mucosa (Tamai et al., 1991) and guinea pig gallbladder (Moummi et al., 1991a), oxidants stimulated the release of PGE_2. Prostaglandins E are well known to produce contraction of intestinal smooth muscle (Eberhart and Dubois, 1995).

Prostaglandins E_1 and E_2 are also reported to release acetylcholine (ACh) from cholinergic neurons (Yagasaki et al., 1981). PGE_1 and PGE_2 increased the excitability of ganglion cells and released ACh from myenteric plexus in longitudinal muscle strip preparations of guinea pig ileum (Yagasaki et al., 1981), and atropine reduced the contraction of guinea pig ileum induced by PGE_1 (Bennett et al., 1968).

Gaginella et al., (1992), also showed that oxidants evoke the release of ACh from rat colonic mucosa/submucosa through a mechanism that at least in part may involve prostaglandins. Because muscarinic stimulation can modulate intestinal contractility, it was speculated that oxidants might produce the observed change in motility through activation of ACh.

However, this seems unlikely in these experiments, because the results showed that atropine did not reduce the contraction induced by OFRs. One explanation is that PGEs may not affect ACh release, as PGEs did not affect ACh release induced by electrical nerve stimulation (Hedqvist et al., 1980). In addition, oxidants might oxidize ACh during or after its release as suggested by Gaginella et al., (1992). This would alter the conclusions regarding the possible significance of liberation of ACh by oxidants (i.e., choline and acetate would be inactive as muscarinic stimulants). Also, it is much easier for atropine to antagonize the effects of exogenously added ACh than to block the effects of ACh released by cholinergic nerve terminals in a close vicinity of the receptor (Diener et al., 1988). Finally, the lack of inhibitory effect of atropine suggests that other transmitters may be involved in the oxidant-induced response.

The present data indicate that pyrilamine, a histamine receptor antagonist, diminishes the DHF/$FeCl_3$-ADP-induced contraction of ileum, suggesting that histamine may play a role in OFRs-induced contraction of ileum. *In vitro* studies

indicate that free radicals liberate histamine from mast cell, either through a selective, noncytotoxic exocytosis of stored granules or through a nonselective, cytotoxic alteration of the plasma membrane (Ohmori et al., 1979; Masini et al., 1987).

Histamine has been shown to be released when the mast cells are preincubated with H_2O_2 (Ohmori et al., 1980). Hydrogen peroxide derived from superoxide radical plays a major role in histamine release (Boros et al., 1991). The less reactive hydrogen peroxide can readily cross membranes and diffuse away from the site of generation. It can interact with reduced state transition metal ions to form hydroxyl radicals which are responsible for histamine release (Boros et al., 1991).

At present the site of histamine liberation is not known, but the participation of mast cells in histamine release is most likely. Upon activation, mast cells can release large quantities of histamine (Metcalfe, 1984). However, the question still remains as to the cellular source of histamine. Additional studies using inhibitors of mast cell secretion are needed to confirm that this cell type is the source of histamine released in the intestine after exposure to DHF/$FeCl_3$-ADP.

Finally, the effects of DHF/$FeCl_3$-ADP could be due to direct effects of oxidants on ileum smooth muscle. In fact, OFRs are strong oxidizing and reducing agents. The oxidation

of sulfhydryl groups in Ca^{2+}-Mg-ATPase significantly decreases ATPase activity (Scutari et al., 1980). Also, oxidation of mitochondrial pyridine nucleotides alters the uptake and release of Ca^{2+} from mitochondria (Scutari et al., 1980). Therefore, OFR-induced oxidation of sulfhydryl groups, resulting in changes in calcium kinetics in ileum smooth muscle mitochondria, SR and / or cell membranes, might constitute the direct mechanism of contraction.

In conclusion, the data of the present study demonstrate that none of the individual inhibitors completely blocked the contracting effect of DHF/$FeCl_3$-ADP. The compartmentalization of the defense system relative to the site of oxidant attack may be an important issue, where even efficient antioxidants are powerless to defend the cell, if the oxidant is generated at a site where it can interact with its target before being degraded. This suggests that factors other than those investigated could also be involved in DHF/$FeCl_3$-ADP-induced contraction of ileum smooth muscle.

7.0 SUMMARY

7.1 Xanthine plus Xanthine Oxidase

In summary, extracellular generation of oxy-radicals by xanthine plus xanthine oxidase resulted in relaxation of ileum. The failure of SOD and catalase to protect ileum from effects of X/XO suggests that superoxide anion and hydrogen peroxide do not play major roles in X/XO-induced relaxation of ileum.

The results of this study suggest that hydrogen peroxide formed extracellularly may enter the cell and interact with intracellular iron to form a highly reactive oxidant. The finding that DMTU and mannitol offered protection against oxy-radical-induced relaxation of ileum suggests formation of hydroxyl radical within the cell. Pretreatment with deferoxamine, a potent iron chelator, reduced the relaxation of ileum, indicating that iron plays an important role in mediating the oxy-radical-induced relaxation of ileum. In addition, the ability of exogenously administered histidine to reduce relaxation suggests that singlet oxygen is also involved in this response.

Xanthine/xanthine oxidase-induced relaxation was dependent on arachidonic acid metabolites, because indomethacin, a cyclooxygenase inhibitor, reduced the

relaxation. The relaxation also appears to be partially dependent on nitric oxide and ATP-sensitive potassium channels, because L-NMMA and glibenclamide partially reduced the relaxation. The xanthine/xanthine oxidase-evoked response in rat ileum was resistant to methylene blue. This suggests that cGMP is not involved in mediating the oxidant responses in stimulated rat ileum.

7.2 Hydrogen Peroxide

Hydrogen peroxide produced concentration-dependent biphasic responses (a transient contraction followed by relaxation) of the ileum. Hydrogen peroxide formed extracellularly may enter the cell and interact with intracellular iron to form a highly reactive oxidant, the hydroxyl radical. The findings that DMTU and mannitol offered protection against H_2O_2-induced biphasic response of ileum suggest formation of hydroxyl radical within the cell.

The contraction and relaxation induced by H_2O_2 was shown to be dependent on arachidonic acid metabolites, because indomethacin reduced the response induced by H_2O_2. Hydrogen peroxide may stimulate the release of arachidonic acid metabolites from a number of potential sources. These metabolites could directly stimulate the smooth muscle to produce changes in motility. In addition, the response was reduced by glibenclamide, suggesting the involvement of prostaglandins in contractions produced by H_2O_2. Hydrogen

peroxide-evoked responses in rat ileum preparation were resistant to L-NMMA, methylene blue, atropine and pyrilamine. This suggests that the neurotransmitters NO, cGMP, acetylcholine and histamine are not involved in mediating the oxidant responses in stimulated rat ileum.

7.3 Dihydroxy Fumaric Acid plus Ferric Chloride and Adenosine Diphosphate

Dihydroxy fumaric acid plus ferric chloride and adenosine diphosphate produced a concentration-dependent contraction of ileum. The ability of mannitol, DMTU or histidine to reduce the contraction suggests the idea that the hydroxyl radical and singlet oxygen are involved in contraction.

The contraction appears to be partly mediated through arachidonic acid metabolites and histamine, because indomethacin and pyrilamine reduced the contraction. Contraction is not mediated by acetylcholine, because atropine did not reduce the response.

8.0 CONCLUSION

The results of this study indicate that the exogenously-generated oxy-radicals produces relaxation, contraction or contraction/relaxation of rat ileum depending upon generating system. Xanthine (X) plus xanthine oxidase (XO) produced relaxation of ileum. The relaxation was attenuated by the hydroxyl radical scavengers, mannitol and dimethylthiourea (DMTU) and the singlet oxygen scavenger, histidine, supporting the idea that the hydroxyl radical (·OH) and the singlet oxygen are involved in X/XO-induced relaxation of ileum. Deferoxamine, an iron chelator, reduced the relaxation of ileum, indicating that ·OH mediates the X/XO-induced relaxation of ileum. Xanthine/xanthine oxidase-induced relaxation is partly mediated through cyclooxygenase metabolites, and partly through nitric oxide and ATP-sensitive potassium channels.

Hydrogen peroxide (H_2O_2) produces a biphasic response (an initial contraction followed by relaxation), which appears to be due to the generation of ·OH. The contraction and relaxation induced by H_2O_2 is mediated through cyclooxygenase metabolites. The hydrogen peroxide-evoked biphasic response in rat ileum is not mediated through nitric oxide, acetylcholine or histamine.

Dihydroxy fumaric acid (DHF) plus ferric chloride ($FeCl_3$) and adenosine diphosphate (ADP) produces concentration-dependent contraction of ileum. Mannitol, DMTU and histidine partially inhibited the DHF/$FeCl_3$-ADP-induced contraction, supporting the idea that hydroxyl radicals and singlet oxygen are involved in DHF/$FeCl_3$-ADP-induced contraction of ileum. DHF/$FeCl_3$-ADP-induced contraction is mediated partly through arachidonic acid metabolites and histamine.

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10.0 APPENDIX

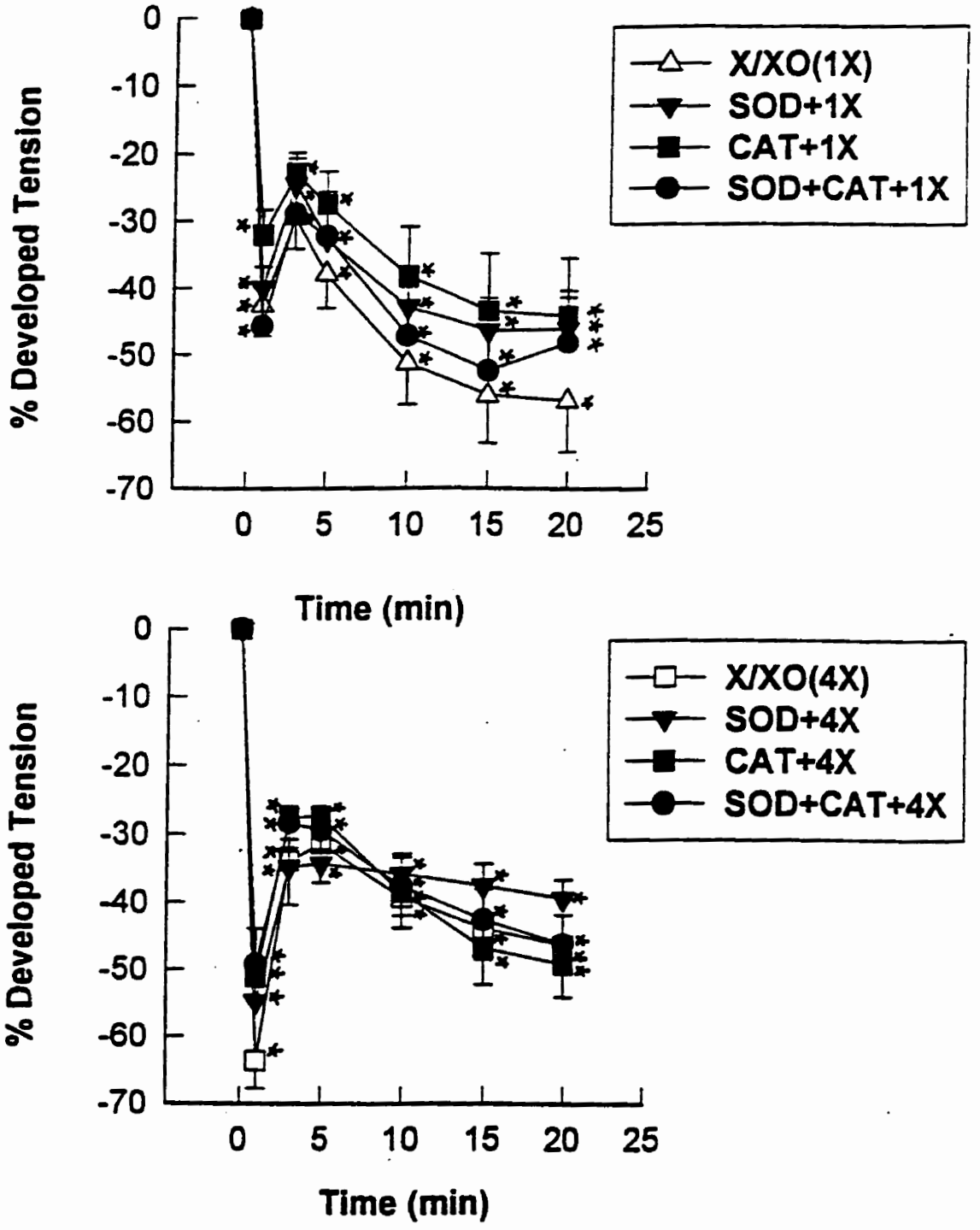


Figure 1: Effects of 1X and 4X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of superoxide dismutase (SOD, 100 U/ml), Catalase (CAT,500 U/ml) and superoxide dismutase plus catalase (SOD+CAT).

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**

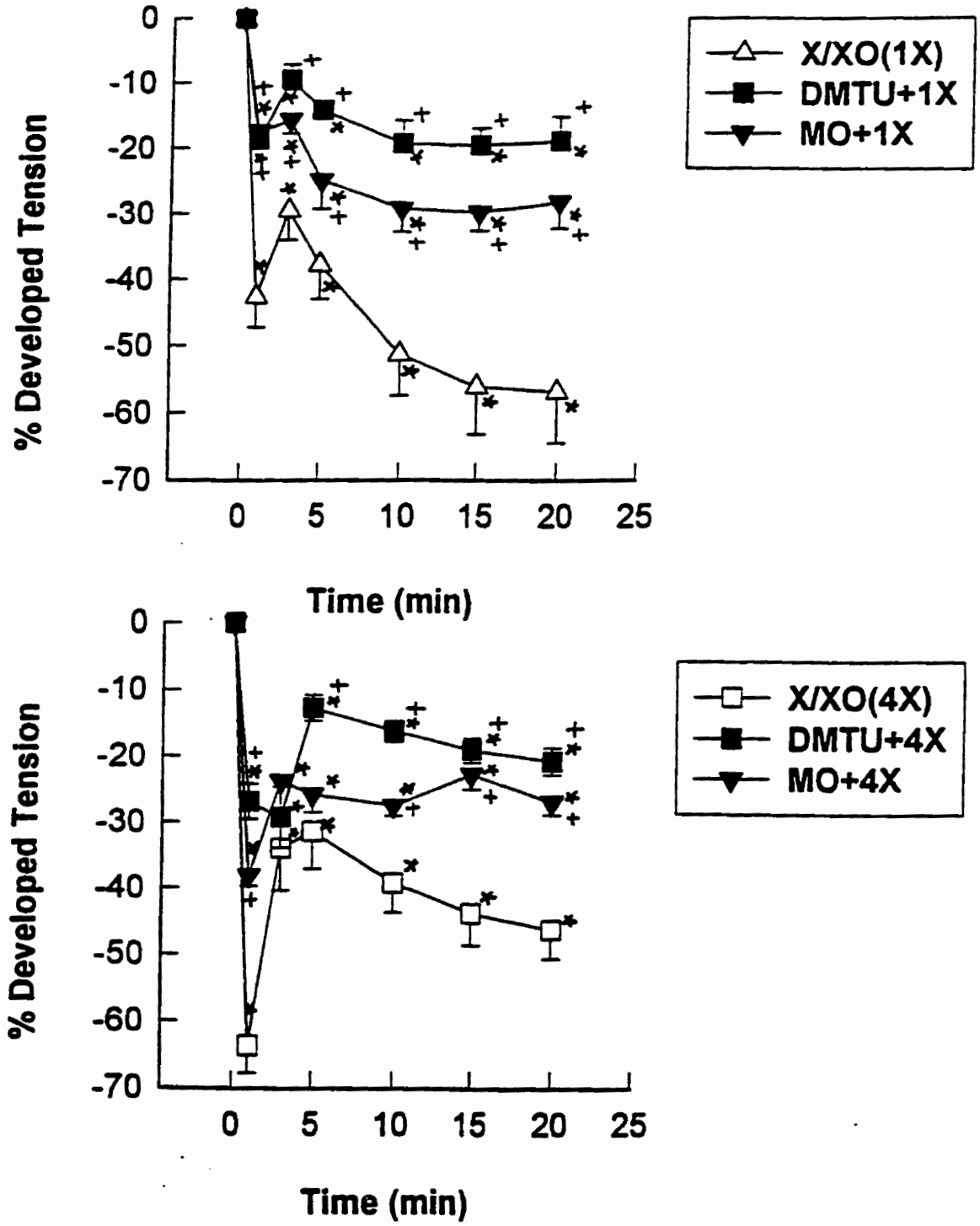


Figure 2: Effects of 1X and 4X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of dimethylthiourea (DMTU) and mannitol (MO) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, X/XO vs DMTU(1.6 mg/ml)+X/XO or MO(80 mM)+X/XO.

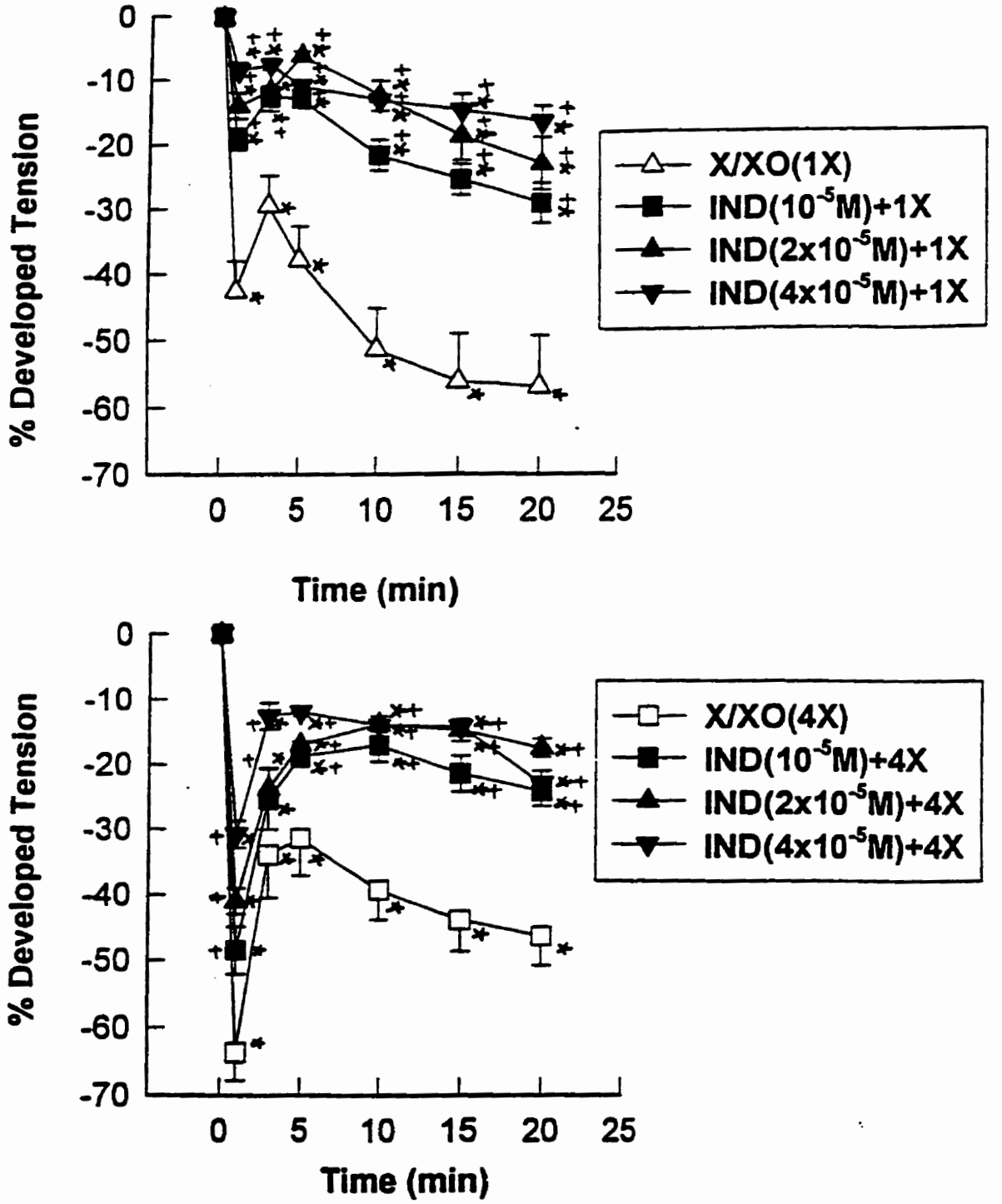


Figure 3: Effects of 1X and 4X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of various concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, X/XO vs IND(10^{-5} M)+X/XO, or IND($2x10^{-5}$ M)+X/XO, or IND($4x10^{-5}$ M)+X/XO.

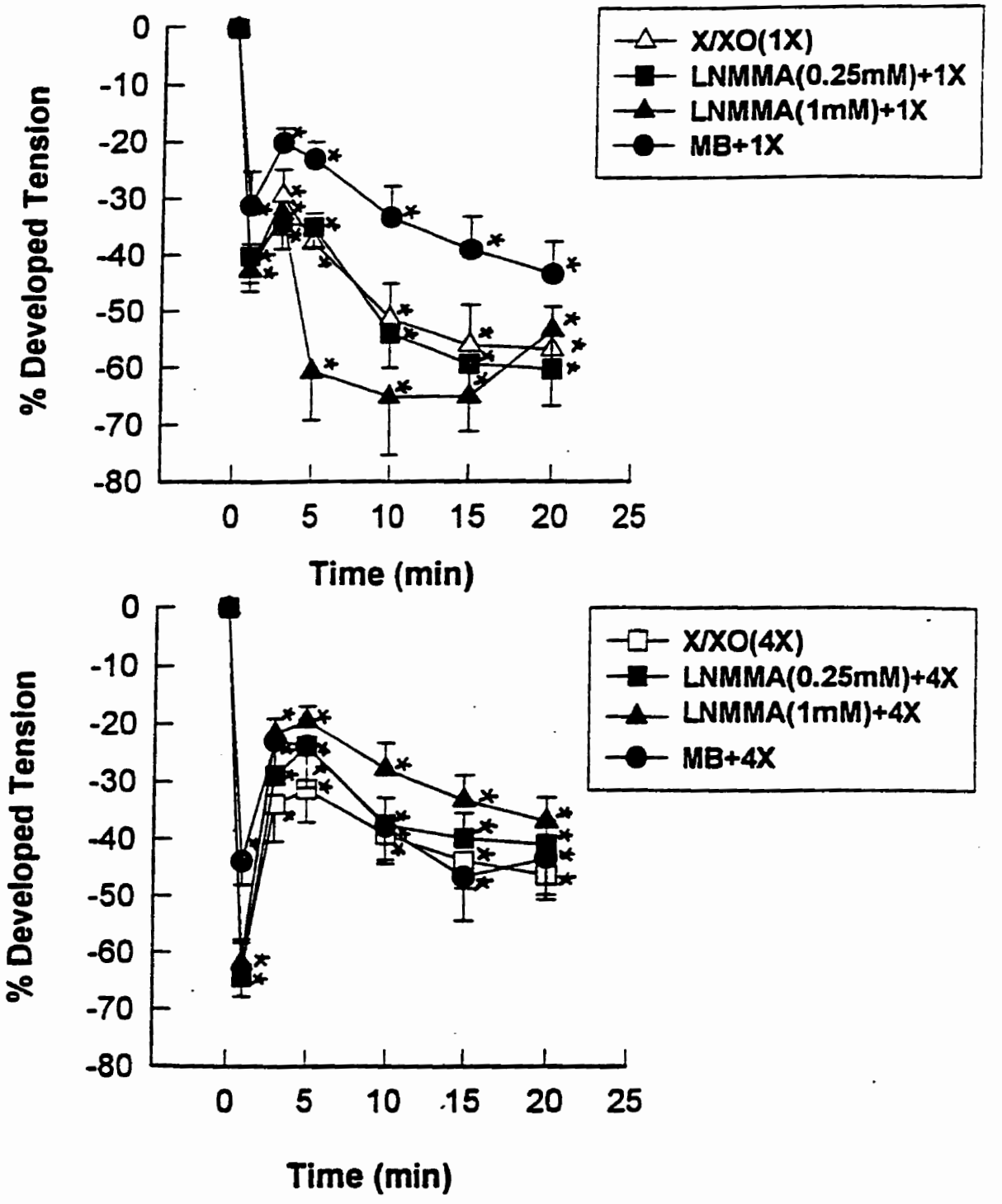


Figure 4: Effects of 1X and 4X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of L-NMMA or methylene blue (MB) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, X/XO vs L-NMMA(1 mM)+X/XO.

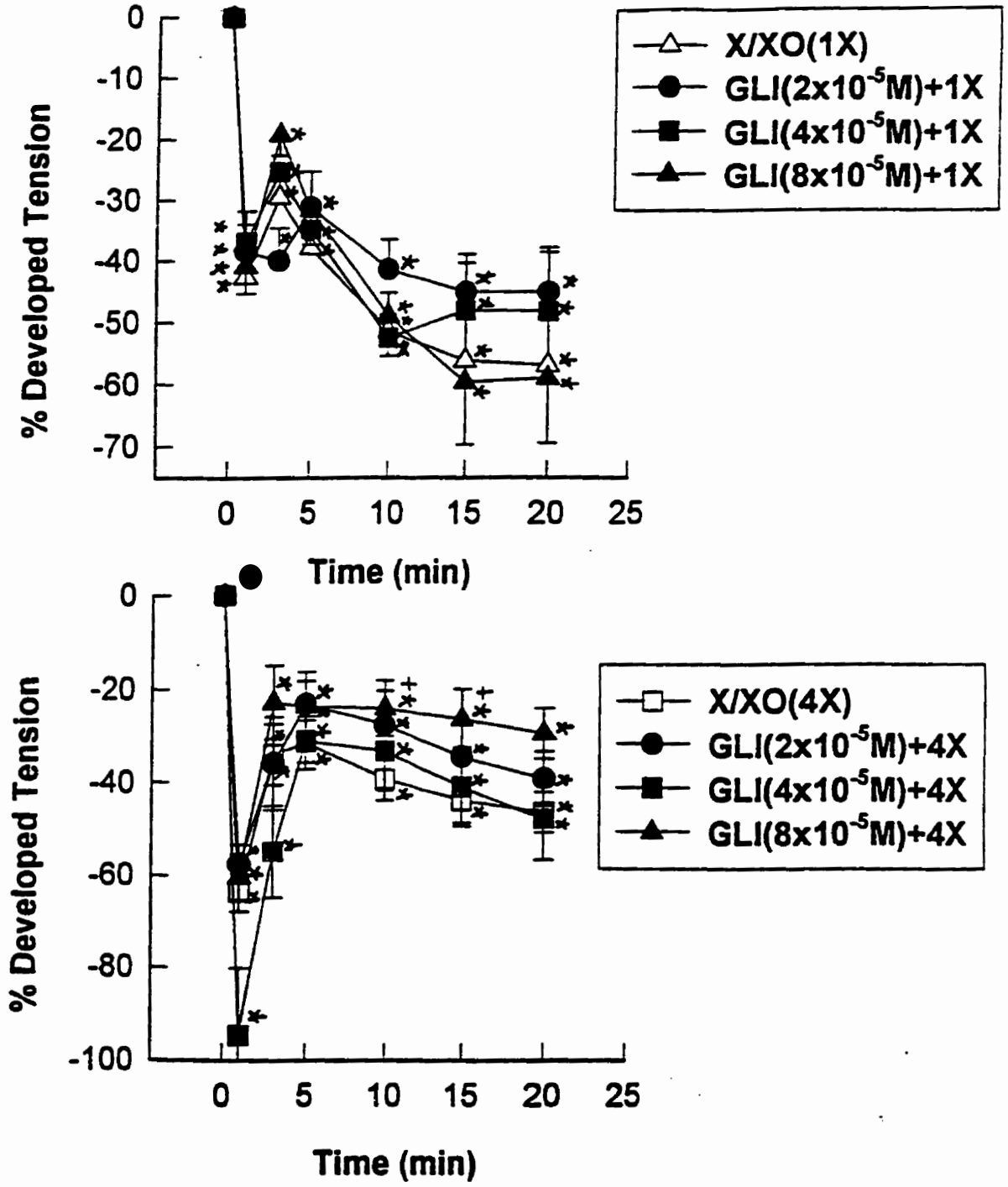


Figure 5: Effects of 1X and 4X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of various concentrations of glibenclamide (GLI) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, X/XO vs GLI($2x10^{-5}$ M)+X/XO, or GLI($4x10^{-5}$ M)+X/XO, or GLI($8x10^{-5}$ M)+X/XO.

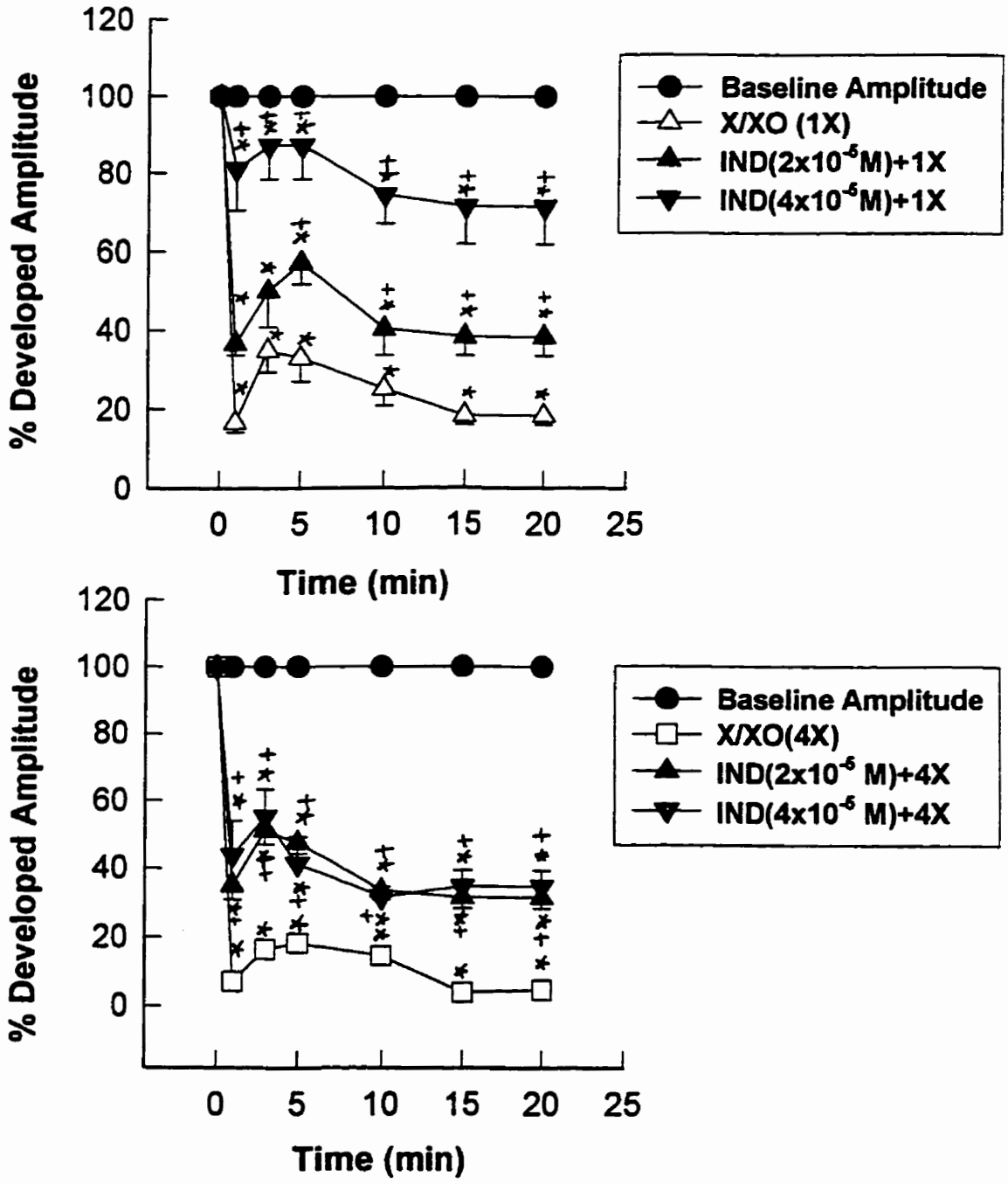


Figure 6: Effects of 1X and 4X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of indomethacin (IND) on amplitude of spontaneous activity of ileum strips.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, Baseline amplitude vs X/XO, or IND($2x10^{-5}$ M)+X/XO, or IND($4x10^{-5}$ M)+X/XO.

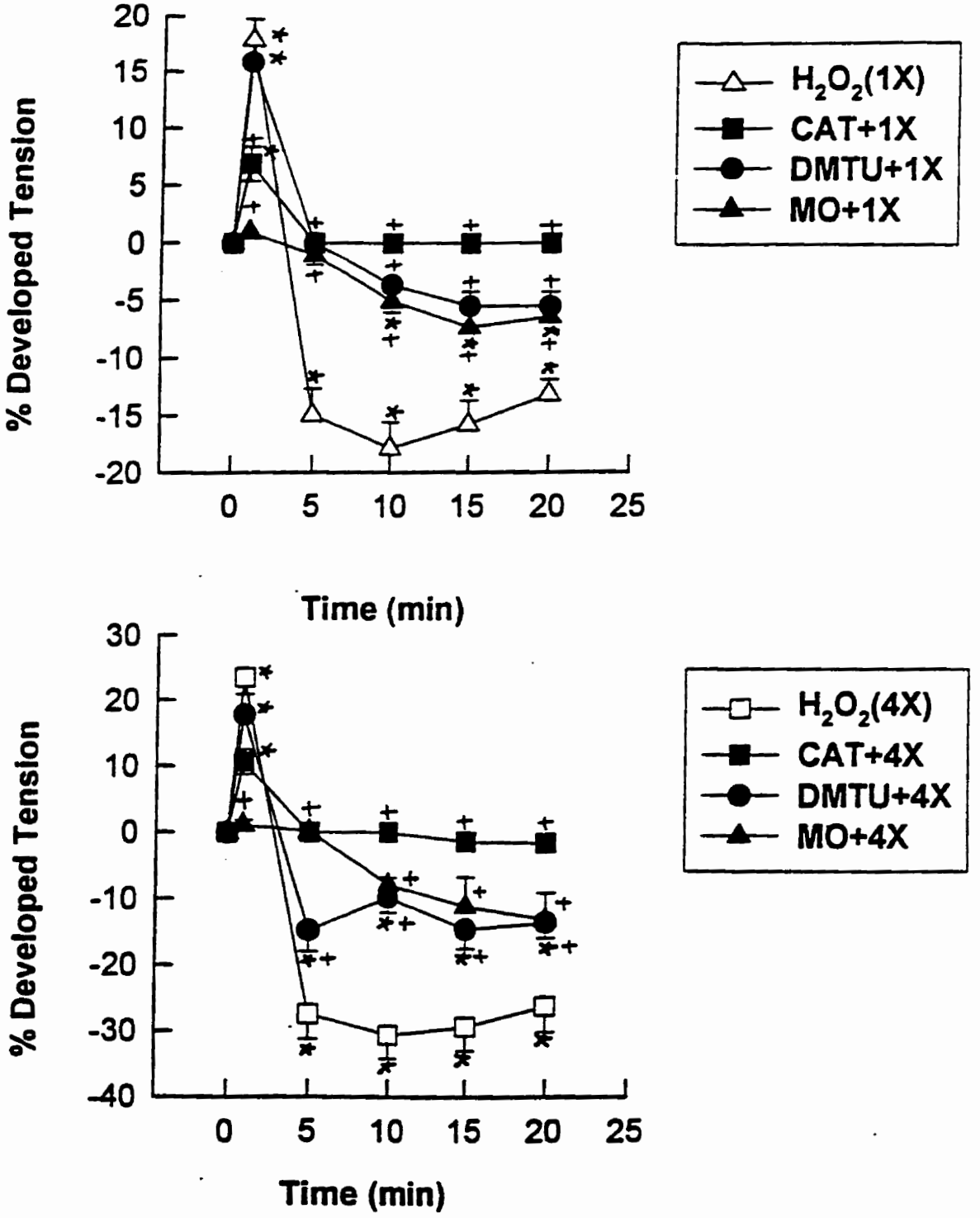


Figure 7: Effects of 1X and 4X concentrations of H_2O_2 in absence or presence of catalase (CAT, 500 U/ml), dimethylthiourea (DMTU, 1.6 mg/ml) or mannitol (MO, 160 mM).

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, H_2O_2 vs CAT+ H_2O_2, or DMTU+H_2O_2, or MO+ H_2O_2.

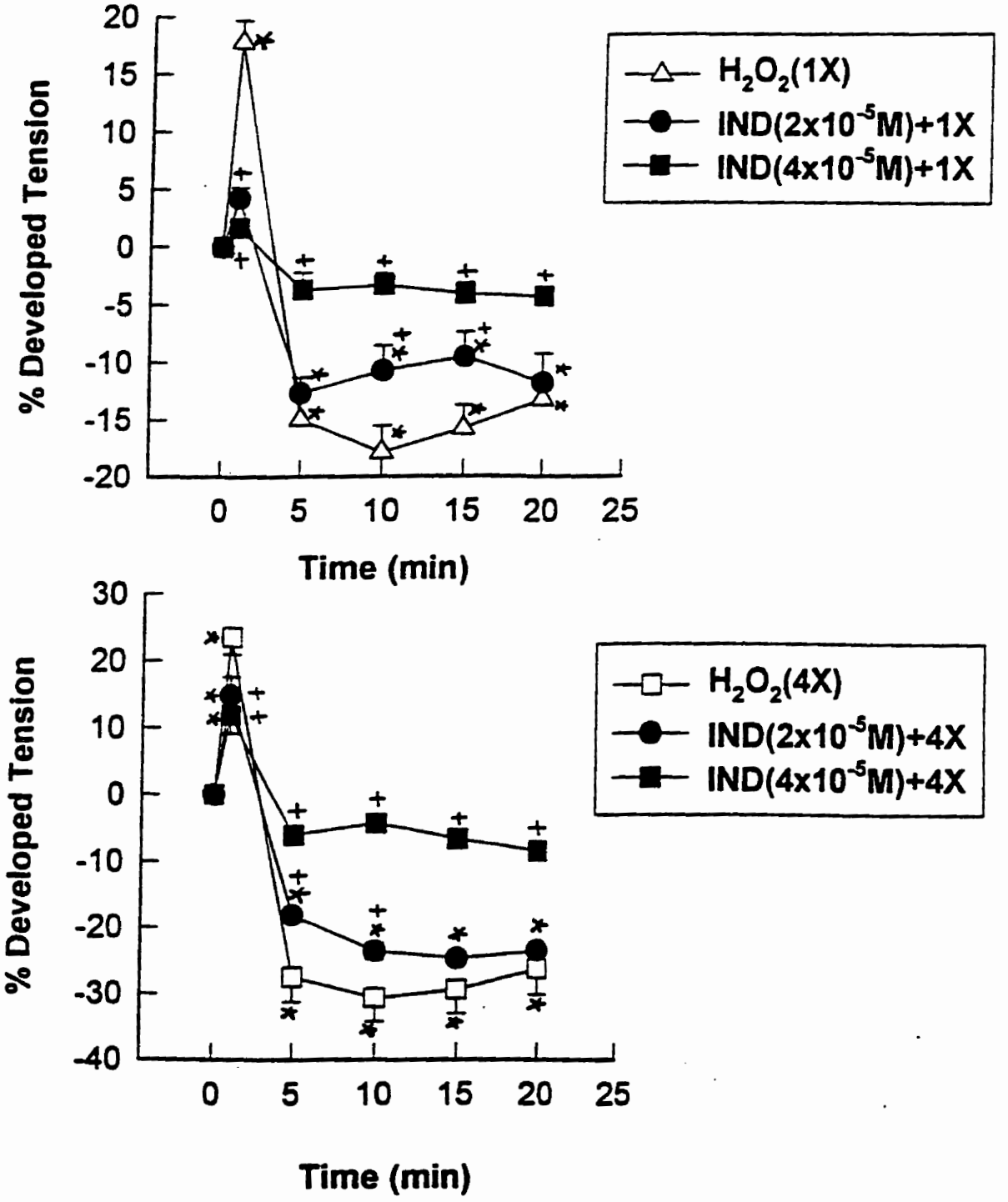


Figure 8: Effects of 1X and 4X concentrations of H_2O_2 in absence or presence of two concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, H_2O_2 vs IND($2x10^{-5}$ M)+ H_2O_2 or IND($4x10^{-5}$ M)+ H_2O_2.

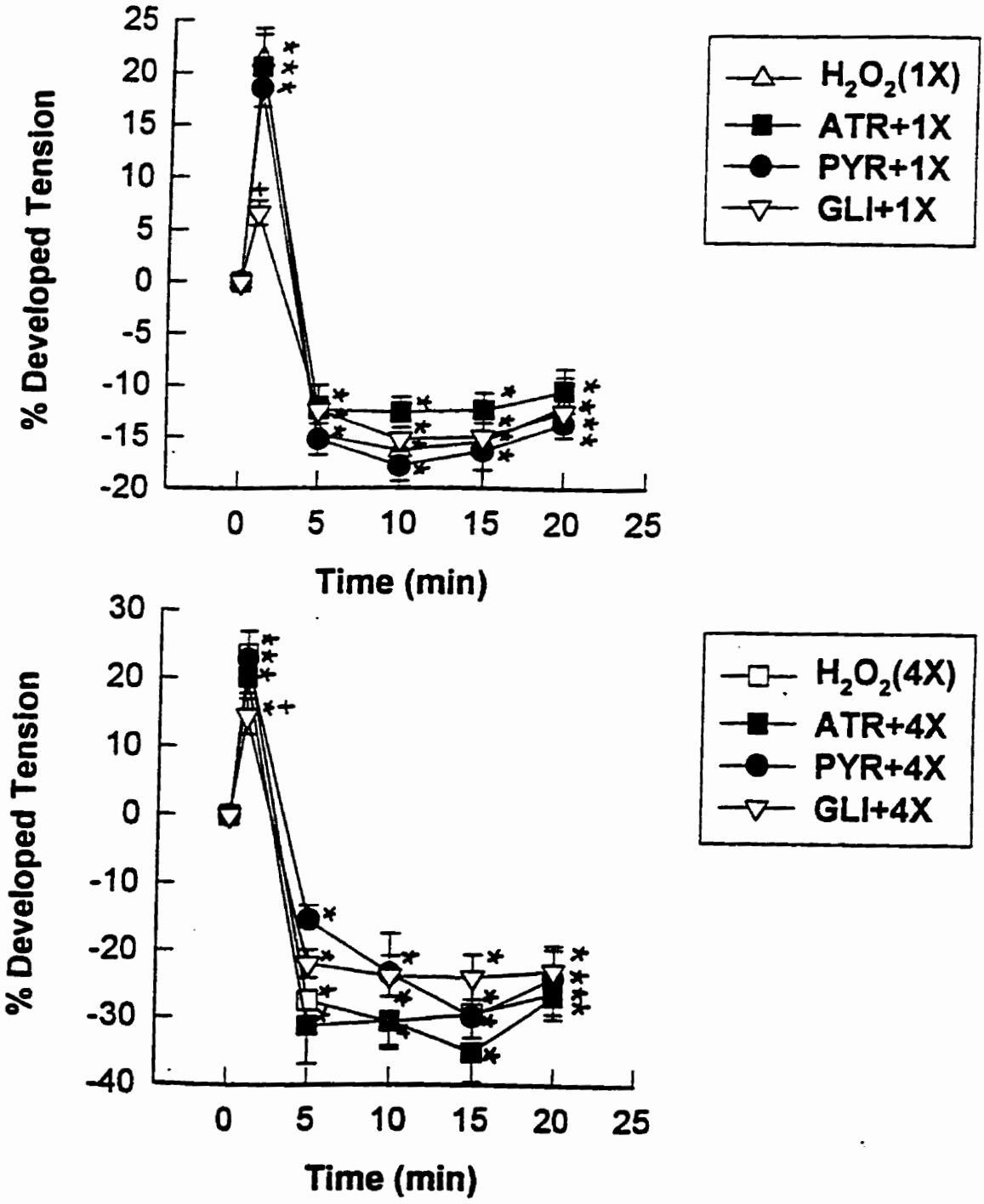


Figure 9: Effects of 1X and 4X concentrations of H_2O_2 in absence or presence of atropine (ATR, 10^{-6} M), pyrilamine (PYR, 10^{-5} M), or glibenclamide (GLI, 10^{-5} M) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, H_2O_2 vs GLI(10^{-5} M)+ H_2O_2.

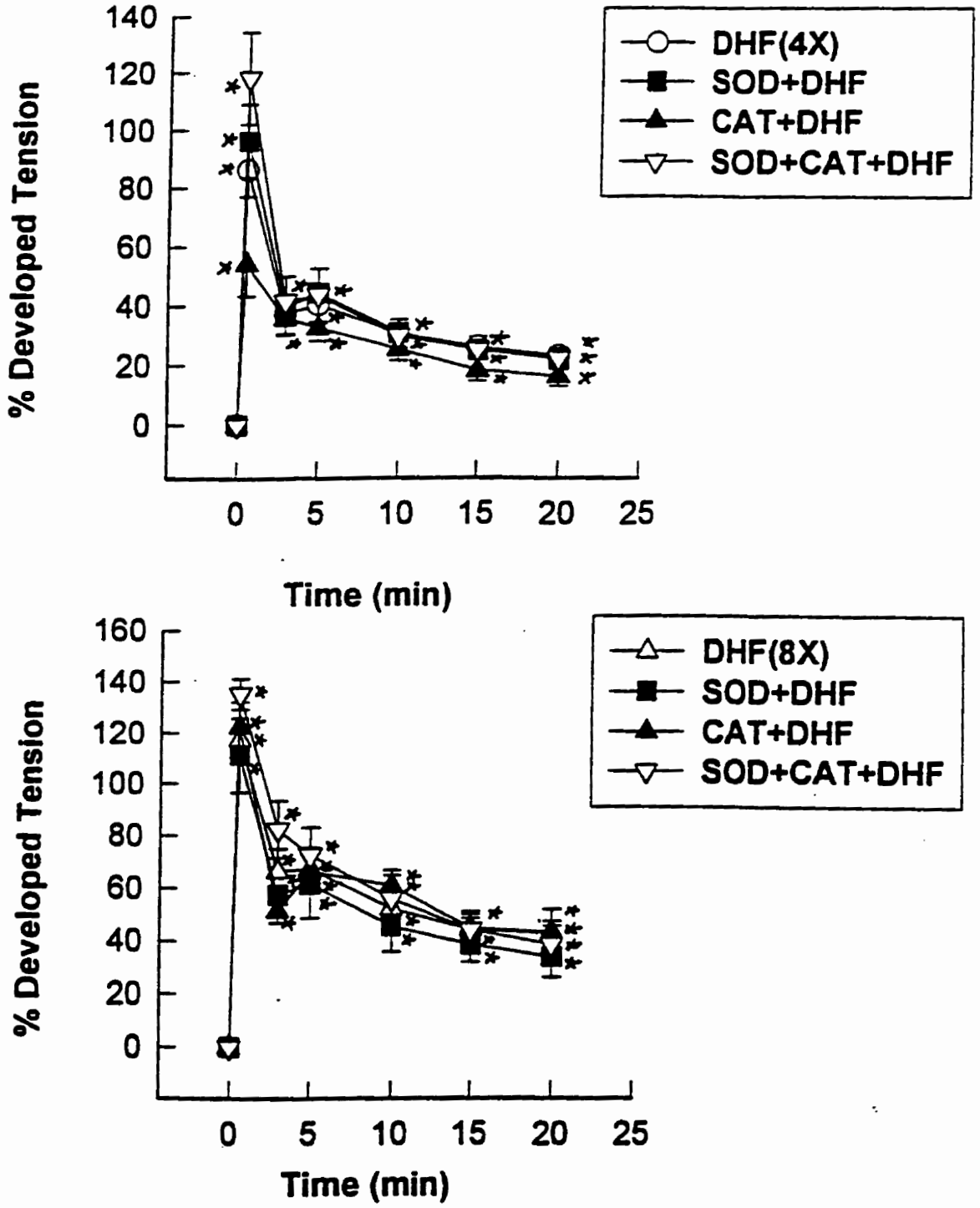


Figure 10: Effects of 4X and 8X concentrations of DHF in absence or presence of superoxide dismutase (SOD, 100 U/ml), catalase (CAT, 500 U/ml) and superoxide dismutase plus catalase (SOD+CAT) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**

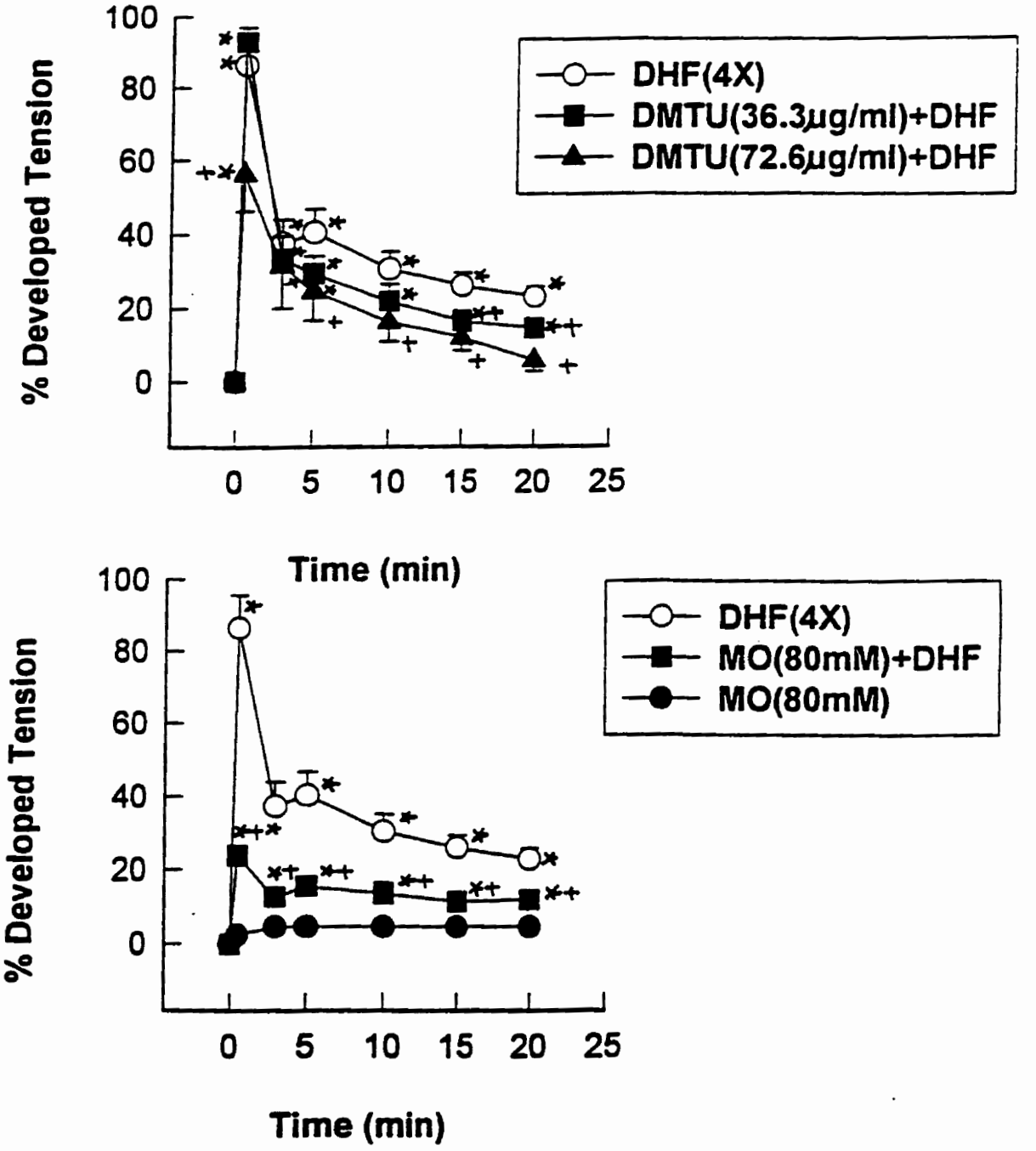


Figure 11: Effects of 4X concentration of DHF in absence or presence of mannitol (MO) or two concentrations of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, DHF vs DMTU(36.3 µg/ml)+DHF, or DMTU(72.6 µg/ml), or MO(80 mM)+DHF.

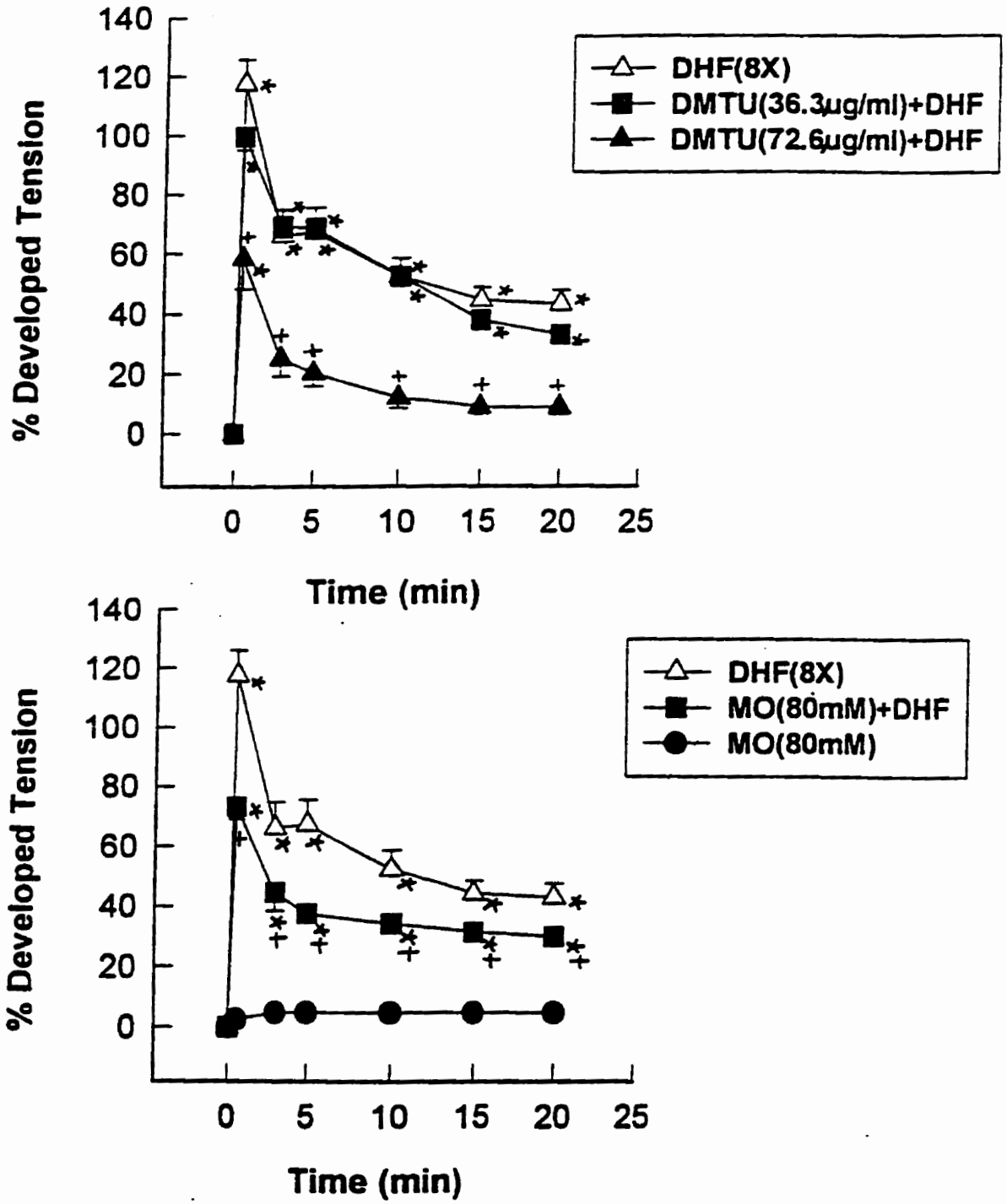


Figure 12: Effects of 8X concentration of DHF in absence or presence of mannitol (MO) or two concentrations of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, DHF vs DMTU(36.3 µg/ml)+DHF, or DMTU(72.6 µg/ml), or MO(80 mM)+DHF.

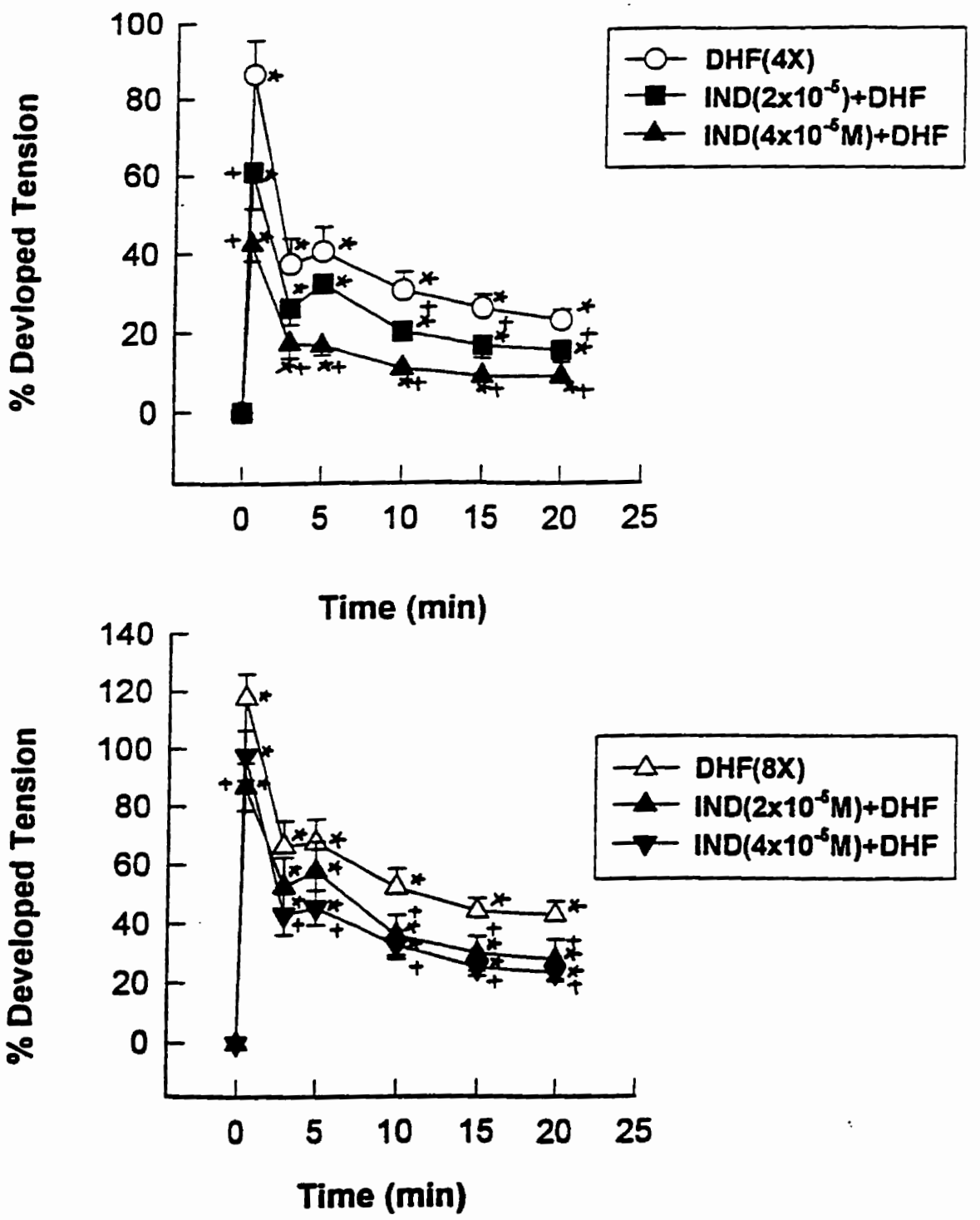


Figure 13: Effects of 4X and 8X concentrations of DHF in absence or presence of two concentrations of indomethacin (IND) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, DHF vs IND($2x10^{-5}$ M)+DHF or IND($4x10^{-5}$ M)+DHF.

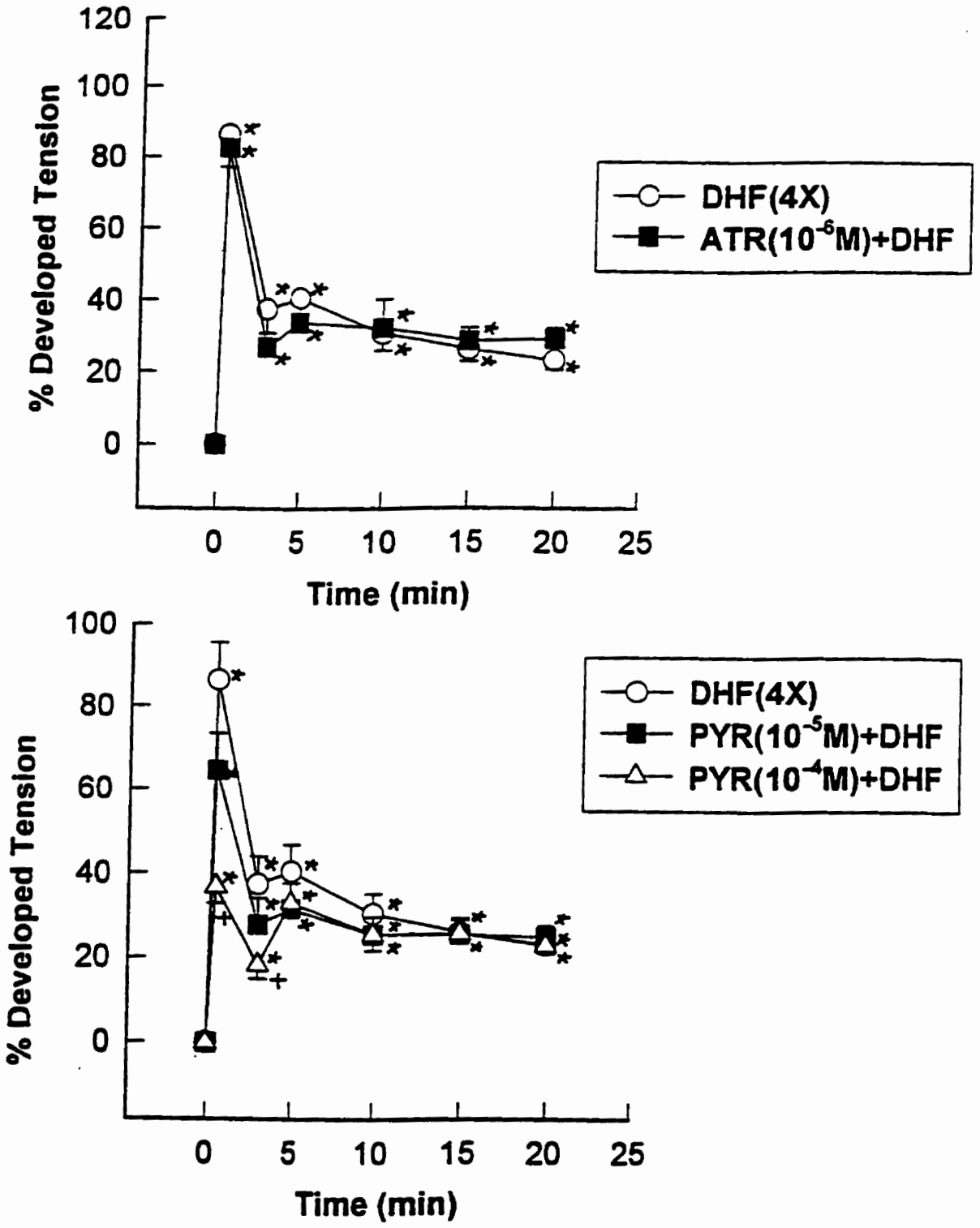


Figure 14: Effects of 4X concentration of DHF in absence or presence of atropine (ATR) or two concentrations of pyrilamine (PYR) on ileum preparations.

Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" time within groups.**
+P<0.05, DHF vs PYR(10^{-5} M)+DHF or PYR(10^{-4} M)+DHF.

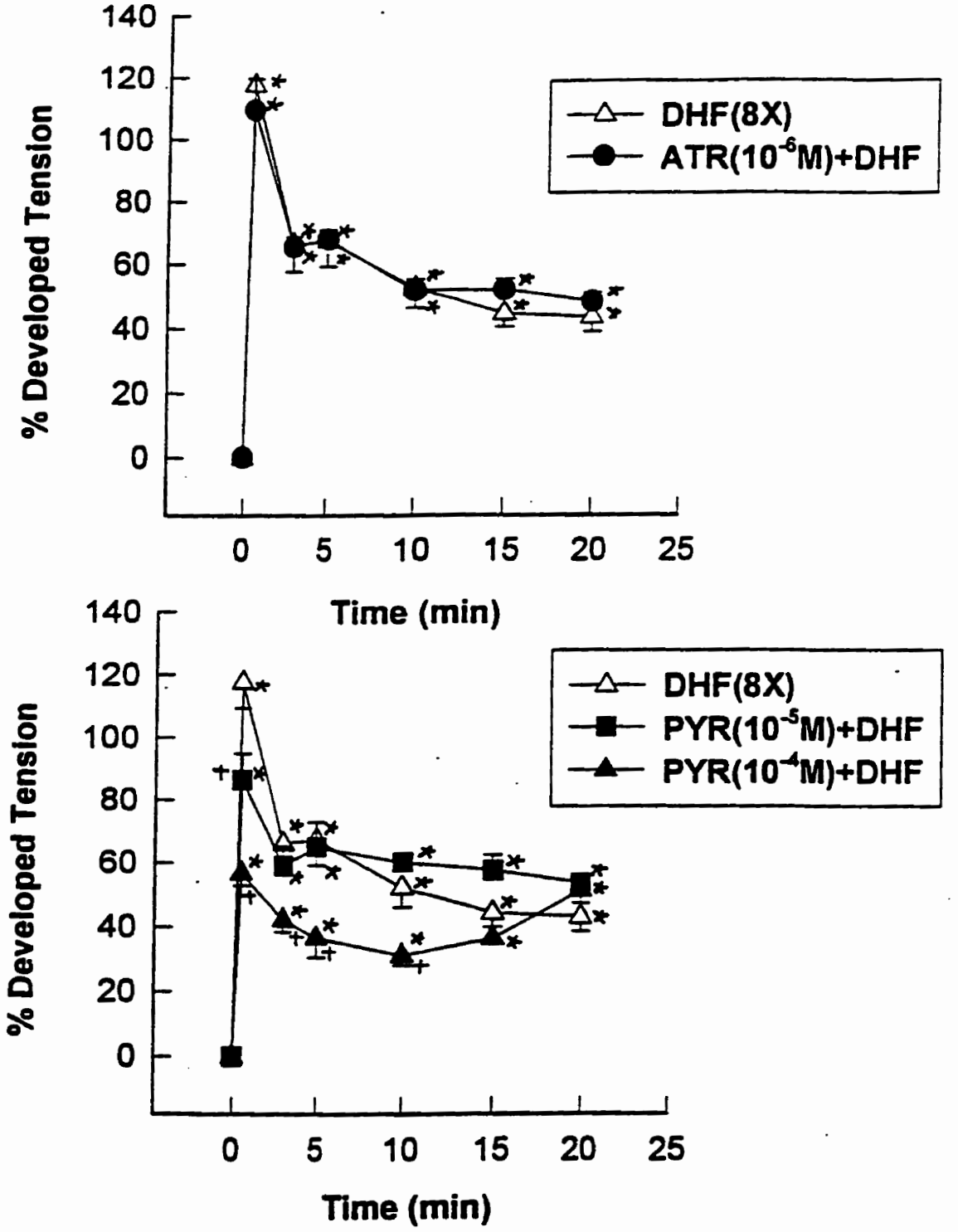
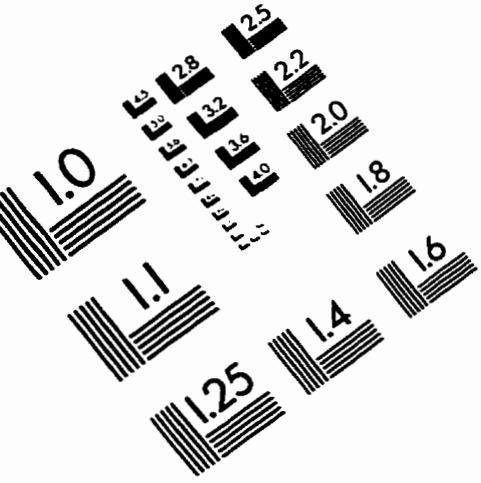
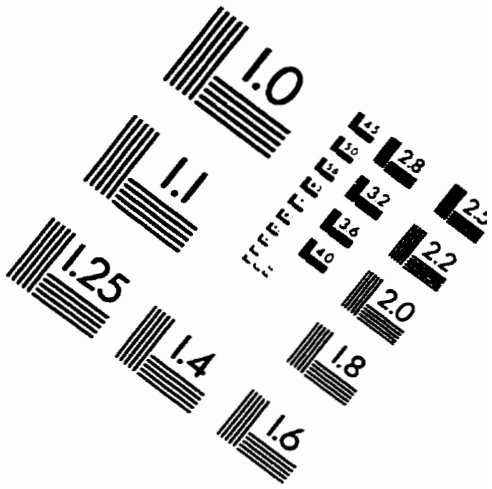


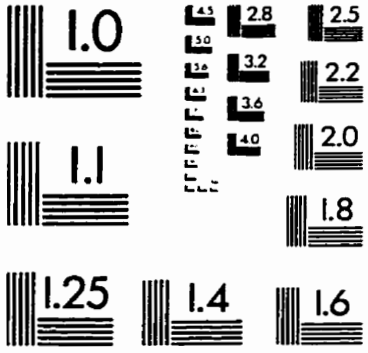
Figure 15: Effects of 8X concentration of DHF in absence or presence of atropine (ATR) or two concentrations of pyrilamine (PYR) on ileum preparations.

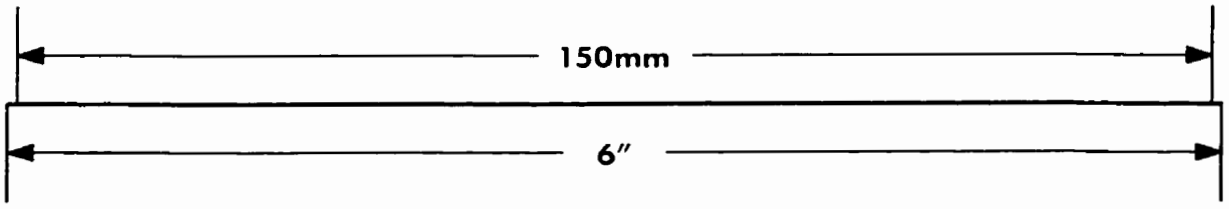
Results are expressed as mean ± S.E.
***P<0.05, comparison of values at different times with respect to values at "0" times within groups.**
+P<0.05, DHF vs PYR(10^{-5} M)+DHF or PYR(10^{-4} M)+DHF.

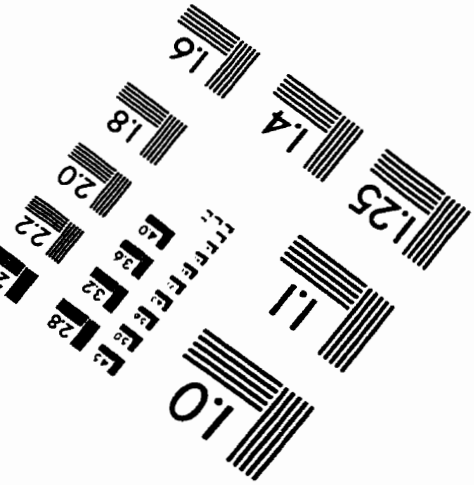
IMAGE EVALUATION
TEST TARGET (QA-3)

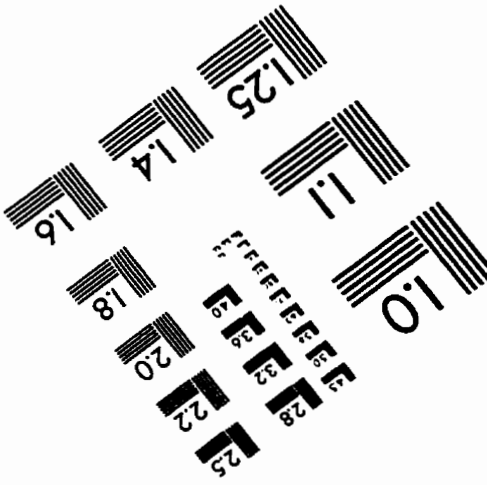












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